Process improvements and techno-economic analyses for the production of short-chain fructose-containing oligosaccharides from sucrose and Jerusalem artichoke tubers

by

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March 2020

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Abstract

Recent years have seen significant growth in the global market for short-chain fructooligosaccharides (scFOS) and inulooligosaccharides (IOS) due to the rising health awareness and demand for calorie controlled foods. The global demand for prebiotics has been estimated at over 200 000 tonnes per year, of which scFOS and IOS constitute a significant fraction. Commercial production of scFOS relies on the enzymatic polymerization of sucrose using β-fructofuranosidase, while the production of IOS relies on the controlled hydrolysis of inulin. South Africa has the potential to contribute its quota to the prebiotic market through the production of scFOS by channelling part of the sugar designated for exportation into the production of this high value product to meet the scFOS local market demand as well as contribute to the international market.

Jerusalem artichoke (*Helianthus tuberosus L.*) tubers have inulin contents similar to that of chicory, coupled with a significant amount of protein. The ability of Jerusalem artichoke (JA) to resist pests and diseases, frost and drought coupled with its ability to grow on most soils with little fertilizer requirements, relieves it of geographical limitations and reduces cultivation expenses. With these unique properties, Jerusalem artichoke has some advantage over chicory. The co-production of IOS and protein, followed by animal feed, ethanol or biogas production from the extraction residues in a biorefinery concept, may improve the economic feasibility of IOS production from Jerusalem artichoke tubers. At present, the main source of inulin exploited for the production of IOS is the roots of chicory. However, the interesting properties of Jerusalem artichoke make it a suitable alternative source of inulin.

The present study sought to improve and compare the economic feasibilities of sucrose and JA tuber as feedstocks for scFOS and IOS production respectively. This involved the minimization of scFOS production cost by exploring and optimizing the different sucrose to scFOS production scenarios (free and immobilized enzyme systems). In objectives 1-3 a novel β-fructofuranosidase responsible for scFOS production from sucrose was immobilized by adsorption onto Amberlite IRA 900 and Dowex marathon MSA anion exchange resins and by entrapment in calcium alginate beads, in the quest to maximise the utilization of the high value enzyme. The data was implemented in objective 4 by simulating three scFOS production

scenarios in Aspen Plus® v8.8 to ascertain the economic feasibility of the free and immobilized enzyme systems of scFOS production by estimating the minimum selling price (MSP) of scFOS. The scFOS production with the free enzyme system resulted in the most profitable scenario with an MSP of 2.61 $/kg compared to the set market price of 5 $/kg.

The optimization of IOS production from various inulin-rich substrates obtainable from JA tubers was carried out in objective 5, with consideration of protein extraction options. The data was applied in objective 6 for the simulation of five multiproduct JA tuber biorefineries in Aspen Plus® v8.8 for IOS, protein, animal feed and bioenergy co-production, as a way of improving the economic feasibility of IOS production from JA tuber. The JA tuber biorefinery for IOS, and animal feed co-production (scenario B) was the most profitable with an MSP of 3.91 $/kg. Comparison of the best-case scenarios of scFOS and IOS productions revealed the free enzyme system of scFOS production from sucrose as the ultimately economically feasible scenario as it required lesser capital investment (15.45 M$ vs 37.82 M$) and operating expenditure (3.40 M$ vs 5.18 M$) with less technical complication than the best case of the JA tuber biorefinery scenarios. The solubility constraints associated with IOS production from the inulin in the JA tuber resulted in increased equipment sizes and utility consumption. Ultimately, the implementation of scFOS and IOS production biorefineries in South Africa would contribute to the South African economy through job creation and revenue generation
.

Opsomming

In die laaste jare is noemenswaardige groei in die globale mark vir kortketting fruktooligosakkariede (scFOS) en inulooligosakkariedes (IOS) as gevolg van die stygende gesondheidsbewustheid en aanvraag vir kalorie-gekontroleerde kosse. Die globale aanvraag vir prebiotika is beraam om meer as 200 000 ton per jaar te wees, waarvan scFOS en IOS ’n merkwaardige gedeelte uitmaak. Kommersiële produksie van scFOS maak staat op die ensimatiese polimerisasie van sukrose deur β-fruktofuranosidase te gebruik, terwyl die produksie van IOS staat maak op die gekontroleerde hidrolise van inulien. Die potensiaal bestaan vir Suid-Afrika om sy kwota by te dra tot die prebiotikamark deur die produksie van scFOS, deur deel van die suiker aangedui vir uitvoer in die produksie van hierdie hoë waarde produk te kanaliseer om aan die scFOS plaaslike markaanvraag te voldoen, sowel as om by te dra tot die internasionale mark.

Aardartisjok (*Helianthus tuberosus L.*) -knolle het inulieninhoud soortgelyk aan die van sigorei, gekoppel aan ’n beduidende hoeveelheid proteïen. Die vermoë van aardartisjok (JA) om peste en siektes, ryp en droogtes te weerstaan, gekoppel aan sy vermoë om op meeste grondsoorte met min kunsmis te groei, verlig dit van geografiese beperkinge en verminder kultiveringsuitgawes. Met hierdie unieke eienskappe het aardartisjok sommige voordele oor sigorei. Die koproduksie van IOS en proteïen, gevolg deur diervoer, etanol of biogasproduksie vanuit die ekstrahering residu in ’n bioraffineerderykonsep, kan die ekonomiese uitvoerbaarheid van IOS-produksie uit aardartisjokknolle verbeter. Tans is die hoofbron van inulien geëksploiteer vir die produksie van IOS die wortels van sigorei. Die interessante eienskappe van aardartisjok maak dit egter ’n gepaste alternatiewe bron van inulien.

Die huidige studie het beoog om die ekonomiese uitvoerbaarheid van sukrose en JA-knolle as voermateriaal vir scFOS- en IOS-produksie, onderskeidelik, te verbeter en vergelyk. Dit het die minimalisering van scFOS-produksiekoste behels deur die verskillende sukrose na scFOS-produksie scenario’s (vry en geimmobiliseerde ensiemstelsels) te ondersoek en optimeer. In doelwitte 1–3 is ’n nuwe β-fruktofuranosidase, verantwoordelik vir scFOS-produksie van sukrose, geimmobiliseer deur adsorpsie op Amberlite IRA 900 en Dowex maraton MSA anioonruilingsharse en deur verstrikking in kalsiumalginaatkrale, in die soektog om die

benutting van die hoë waarde ensiem te maksimeer. Die data is geïmplimenteer in doelwit 4 deur drie scFOS-produksie scenario's in Aspen Plus® v8.8 te simuleer om die ekonomiese uitvoerbaarheid van die vry en geimmobiliseerde ensiemstelses van scFOS-produksie te bepaal deur die minimum verkoopsprys (MSP) van scFOS te beraam. Die scFOS-produksie met die vry ensiemstelsel het die mees winsgewende scenario tot gevolg gehad met 'n MSP van 2.61 $/kg in vergelyking met die vaste markprys van 5 $/kg.

Die optimering van IOS-produksie van verskeie inulienryke substrate verkrygbaar uit JA-knolle is in doelwit 5 uitgevoer, met inagneming van proteïenekstraheeropsies. Die data is toegepas in doelwit 6 vir die simulasie van vyf multiproduk JA-knol-bioraffineerderye in Aspen Plus® v8.8 vir IOS, proteïen, diervoer en bioenergie koproduksie, as 'n manier om die ekonomiese uitvoerbaarheid van IOS-produksie uit JA-knolle te verbeter. Die JA-knolbioraffineerdery vir IOS, en diervoer koproduksie (scenario B) was die winsgewendste met 'n MSP van 3.91 $/kg. Vergelyking van die beste geval scenario's van scFOS- en IOS-produksies het gewys dat die vry ensiemstelsel van scFOS-produksie uit sukrose die eindelike ekonomiese uitvoerbare scenario is, omdat dit minder kapitaalbelegging (15.45 M$ vs. 37.82 M$) en bedryfsuitgawes (3.40 M$ vs. 5.18 M$) benodig, met minder tegniese komplikasies as die beste geval van die JA-knolbioraffineerdery-scenario's. Die oplosbaarheidbeperkinge geassosieer met IOS-produksie uit die inulien in die JA-knol het in verhoogde toerusting groottes en utiliteitgebruik gelei. Eindelik sal die implimentering van scSOF en IOS-produksie bioraffineerderye in Suid-Afrika bydra tot die Suid-Afrikaanse ekonomie deur werkskepping en inkomste generasie.

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Nomenclature and abbreviations

A_e	Number of activity units detected on the equivalent amount of free enzyme
A_f	Number of activity units found in the filtrates and washing solutions after immobilization
A_i	Total number of activity units of the starting enzyme solution used for the immobilization process
AI900	Amberlite IRA 900
AIE	Amberlite IRA 900 immobilized enzyme
A_m	Number of activity units on the support material after immobilization and washing
A_r	Activity recovery
CA	Calcium alginate
CAIE	Calcium alginate immobilized enzyme
DCFROR	Discounted cash flow rate of return
DHSFRM	Solid standard enthalpy of formation
DMM	Dowex Marathon MSA
DP	Degree of polymerization
DTT	Dithiolthreitol
E_f	Immobilization efficiency
F	Fructose
F2	Inulobiose
F3	Inulotriose
F4	Inulotetraose
F5	Inulopentaose
FCI	Fixed capital investment
FE	Free enzyme
FFASE	β-fructofuranosidase
FOC	Fixed operating cost
G	Glucose
GF	Sucrose

GF2	1-Kestose
GF3	Nystose
GF4	1^F-fructofuranosylnystose
ha	Hectare
HPLC	High Performance Liquid Chromatography
IOS	Inulooligosaccharides
IRR	Internal rate of return
JA	Jerusalem artichoke
kW	kilowatt
kWh	Kilowatt-hour
M$	Million US dollars
MSP	Minimum selling price
MW	Molecular Weight
NPV	Net present value
OD	Optical density
rpm	Rotations per minute
scFOS	Short-chain fructooligosaccharides
SMB	Simulated moving bed chromatography
tpa	Tonnes per annum
TCI	Total capital investment
TEPC	Total equipment purchase cost
TOC	Total operating cost
TPDC	Total plant direct cost (TPDC)
TPIC	Total plant indirect cost
WC	Working capital
w/w	weight per weight
w/v	weight per volume
Y_i	Immobilization yield

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Chapter 1

1 Introduction

1.1 Background

Short-chain fructose-containing oligosaccharides consisting of short-chain oligosaccharides (scFOS) and inulooligosaccharides (IOS) make up 10% of the market for natural sweeteners, which has seen significant growth in recent years. The world market for functional foods is well above $33 billion with US, Europe and Japan contributing the greatest fractions [1]. The demand for prebiotics which constitute a small fraction of the functional foods market has been growing rapidly [1]. This can be attributed to the increasing awareness of health concerns such as diabetes, obesity, and atherosclerosis, coupled with the surge in demand for functional and calorie-controlled foods [2], [3]. The most popular components of the prebiotics are the short-chain fructose-containing oligosaccharides namely short-chain fructooligosaccharides (scFOS) and inulooligosaccharides (IOS). These two are competing products in the marketplace as they are known to express similar functionalities when included in the human diet [3].

Global Industry Analysts Inc. (GIA) estimated that the U.S. market for prebiotics would reach the $225.1 million mark by 2015, while that of the European market would reach $1.17 billion [4]. Japan also holds a sizeable share of the prebiotics market with a prebiotic oligosaccharides demand of 69 000 tonnes/year of which short-chain fructose-containing oligosaccharides make up about 6.5% [1], [5]. The production of prebiotics in Europe is estimated at 30 000 tonnes/year [1]. At present, the global demand for prebiotics is estimated to be around 200 000 tonnes [6]. The short-chain fructose-containing oligosaccharides market is prominent in East Asia, Europe and North America with estimated annual growth rates of 15% [1]. In Japan alone the demand for these fructose-containing prebiotics exceed local production levels by 29% indicating the need for new players in the market [5], [7]. Protein-based nutraceuticals also have a large share in the revenue of nutraceuticals globally. In 2011, their market revenue was estimated at $3.6 billion [8]. Protein nutraceuticals can be consumed as nutritional supplements or used in processing a variety of functional foods, due

to their low cholesterol and calorie contents, in addition to their health promoting properties, as compared to egg and milk-based proteins [9].

To meet the increasing demand of the short-chain fructose-containing oligosaccharides, a number of companies emerged as manufacturers, either by the enzymatic polymerization of sucrose or the enzymatic hydrolysis of inulin. Beghin-Meiji industries of France produces scFOS from sucrose under the trade name of Actilight® [6]. Actilight has gained popularity in over 150 countries worldwide due to its quality and proven performance [8]. Orafti Active food ingredients, USA produces inulin and IOS from chicory roots under the trade names Raftiline® and Raftilose® respectively [10], [11]. It is important to note that the term IOS was only used in this dissertation when exclusively referring to the fructooligosaccharides from inulin and the term scFOS was used when discussing the fructooligosaccharides from sucrose alone. Collectively, they were referred to as short-chain fructose-containing oligosaccharides.

South Africa has the potential to contribute its quota to the scFOS market through the production of scFOS from the significant amounts of sugar produced annually. A consistent annual sugar production of about 2.2 million tonnes has been achieved over the past 5 years of which 40% is exported [12]. Part of the sugar designated for export could be channeled into the production of this high value product to meet the scFOS demands of the local and international markets. This would help to increase the total income of R8 billion per annum obtained from direct sales of sugar locally and internationally. scFOS as alternative sweetener to sucrose is particularly attractive in the local market due to the health promotion levy applied to sucrose-sweetened products.

scFOS production from sucrose provides an avenue to add more value to industrial and food grade sugar. However, the resulting yields are usually around 60% ($w_{FOS}/w_{sucrose}$) due to glucose inhibition [13]. Much research has been directed towards alleviating that effect [14]–[16]. Using the free enzyme system for scFOS production is by far the process that assures high scFOS yields [17], but the main drawback is that the enzyme is only used once and then discarded during purification of scFOS. The recovery of the free enzyme for re-use in subsequent reaction batches is technically challenging and expensive. Immobilization presents the solution by enhancing enzyme stability and also providing the possibility of recovery and re-use of these expensive enzymes. Immobilization also allows for easy handling

of enzymes especially in an industrial process since the immobilized enzymes become less sensitive to minor fluctuations in temperature and pH, which increases the stability of the catalytic activity [18].

Jerusalem artichoke (*Helianthus tuberosus* L.) tubers have inulin contents (65 - 80% dry weight) similar to that of chicory (average of 68% dry weight), coupled with a significant amount of protein (15 - 16% dry weight) [19], [20]. Jerusalem artichoke demonstrates high resistance to pests and diseases, frost and drought coupled with high growth rates in most soils with little fertilizer requirements [21]–[23]. With these unique properties, Jerusalem artichoke has some advantages over chicory. The co-production of IOS and protein, followed by ethanol or biogas production from extraction residues in a biorefinery concept, may improve the economic feasibility of IOS production from Jerusalem artichoke tubers [24]. The potential use of Jerusalem artichoke for sustainable bioproducts production should not inhibit the production of food, as it is not considered as a conventional food crop and its cultivation does not necessarily require the use of arable land with high irrigation and fertilizer demand [25]. At present, the main source of inulin exploited for the production of IOS is the roots of chicory. However, the properties of Jerusalem artichoke make it a suitable alternative source of inulin and consequently IOS [11].

1.2 Motivation for study

The β-fructofuranosidase enzyme used for sucrose conversion to scFOS, which is of interest in this study, has been engineered to relieve the effect of substrate inhibition and improve its thermostability, allowing for high sucrose conversion and consequently high scFOS yields [16]. However, the challenge of underutilization of the enzyme in soluble form still persists. This study sought to explore the various ways by which this enzyme could be immobilized and further tested the performance against the free enzyme counterpart since immobilization has been reported to offer some technical benefits to the enzyme. As a result of the technical and functional advantages of immobilization over the free enzyme reported in literature, most authors have proposed that the industrial application of an immobilized β-fructofuranosidase in scFOS production may offer potential economic advantages [18], [26]–[30]. However, to the best of the author's knowledge, an actual economic study comparing the immobilized

enzyme and free enzyme systems was yet to be fully explored. This study contributed to filling that gap.

There is a considerable amount of literature on Jerusalem artichoke (JA) that highlights the potential of the tuber as feedstock for plant-based inulin, which is a precursor for the production of many compounds, such as L-lactic acid, acetone-butanol, 2,3-butanediol, butyric, succinic acid, sorbitol, biodiesel, ethanol, inulooligosaccharides and protein. Some studies have focused on the use of inulin for the production of ethanol. The tubers also contain a significant amount of protein. It is anticipated that greater value could be obtained from the Jerusalem artichoke tuber by isolating protein as a separate product, converting inulin to IOS, and producing lower value commodities like ethanol and biogas from the residues of these processes. The utilization of the JA tuber biomass by co-production of IOS, protein and bioenergy in a biorefinery concept was therefore considered. The extraction of inulin and proteins from the tubers usually leaves some portions of these components in the residues from these processes [31], [32]. The residual inulin in the waste materials provide a suitable feedstock for ethanol or biogas production, providing opportunity to improve the sustainability and economic viability of the biorefinery. The residues containing residual unconverted monomer sugars and fats and oils have the potential to be used as animal feed providing additional revenue.

Data on the co-production production of IOS and protein from Jerusalem artichoke tubers, coupled with biofuel production from the residues, and the use of the residues as livestock feed is virtually non-existent. Neither has a proper economic evaluation of such a biorefinery application of the Jerusalem artichoke tuber been conducted, especially considering the effective IOS production cost in such a biorefinery, compared to the cost of scFOS production from sucrose. The proposed project sought to fill that gap by optimizing a process to maximize IOS production alongside protein extraction from the JA tubers. Much attention directed to the IOS production process due to the complexity of the process. The study further explored the potential of IOS, protein and biofuel production from the JA tubers in a biorefinery concept. This approach to IOS production was compared to options for scFOS production from sucrose, to determine which approach has greater economic viability. The production cost of scFOS from sucrose is partly determined by the selection of an enzyme

system for its synthesis, for which both free and immobilised enzymes were considered. Economic models were developed for the various IOS, protein, ethanol and biogas production sequences from JA tubers and ultimately, were compared to that of scFOS production from sucrose to identify the most attractive investment scenario.

The competitive nature of the scFOS and IOS in the marketplace required that production processes of both products be considered for improvements and optimizations by means of experimentation to provide the platform for a fair comparative techno-economic assessment for both products. Considering that South Africa has the potential to contribute to the prebiotic market by commercial production of either scFOS or IOS, the techno-economic evaluation of both products provides the opportunity to determine which product would be more economically beneficial to the economy since they have identical functionalities.

1.3 Structure of dissertation and summary of novel contribution

After the introduction in Chapter 1, the literature review in Chapter 2 provides detailed information on scFOS and IOS and their production from sucrose and inulin respectively. Immobilization of the high value β-fructofuranosidase was also reviewed in the quest to improve the economics of scFOS production. Finally, the prospects of JA as a biorefinery crop was also reviewed in detail. Chapter 3 outlines the aim and objectives of the study. Chapters 4 – 7 are individual studies, which have been prepared in article format for publication. In Figure 1.1, the relationship between the objectives and the respective work chapters is presented together with the summary of novel contribution of each work chapter. A comparison of the technical and economic advantages of the best-cases from the scFOS and IOS scenarios are also detailed in Chapter 7. The summary of the main conclusions and the recommendations are outlined in Chapter 8.

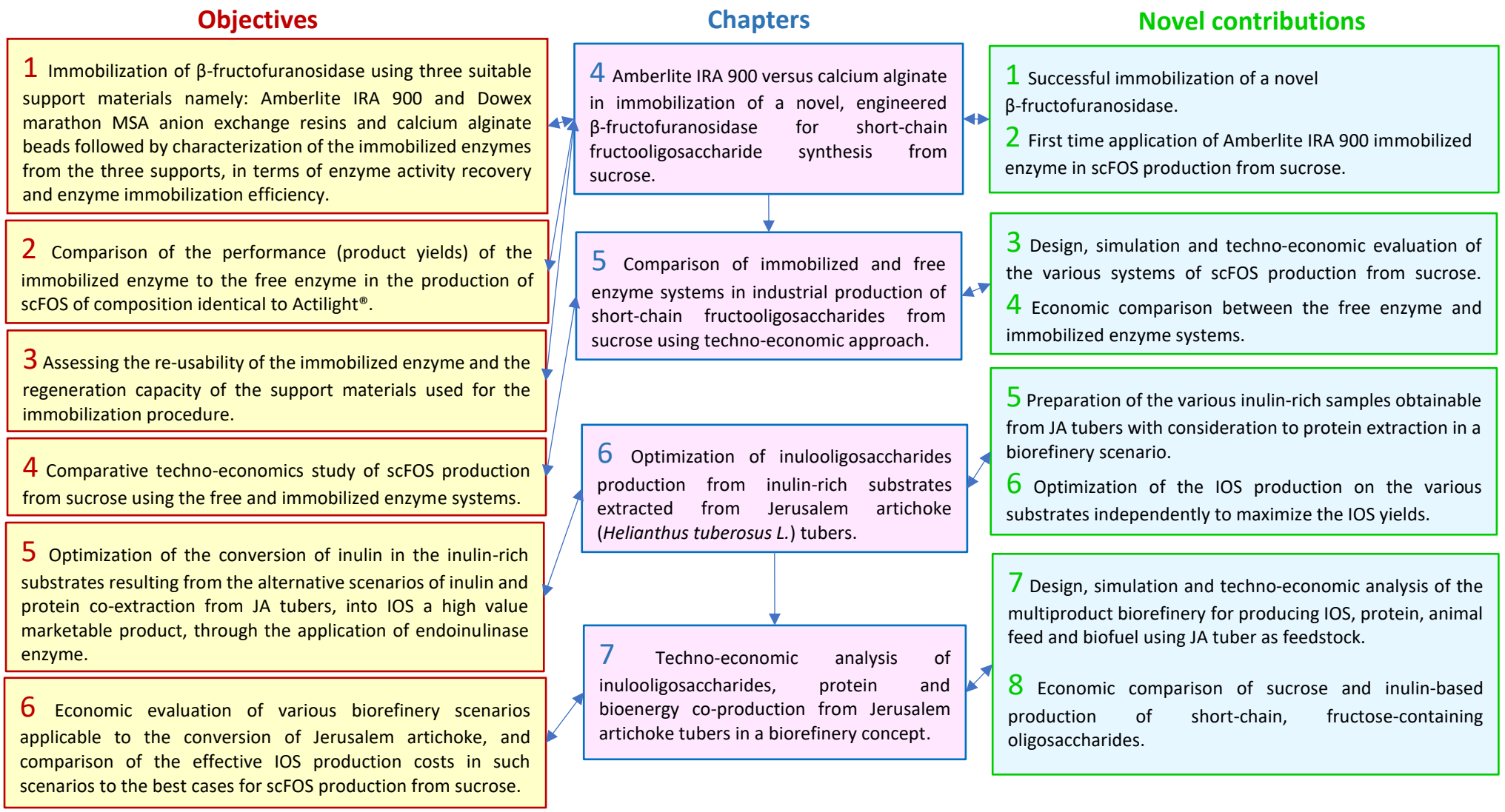

Figure 1.1: Outline and novel contribution of work chapters

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Chapter 2

2 Literature review

2.1 Short chain fructooligosaccharides and inulooligosaccharides

Short-chain fructooligosaccharides (scFOS) and inulooligosaccharides (IOS) are short-chain fructose-containing oligosaccharides that have attracted much attention due to the increased demand for healthier and calorie-controlled sweeteners. This upsurge is attributed to the rising health consciousness. These calorie-controlled sweeteners have been introduced into the market for usage in situations where their conventional counterparts mainly sucrose and high fructose corn syrup (HFCS) are not applicable [1]. They are especially attractive due to the prebiotic and health benefits that they provide to humans and animals, in addition to being sweeteners [2].

2.2 Composition, properties and applications

Short chain fructooligosaccharides (scFOS) consists of 1-kestose (GF2), nystose (GF3) and 1^F-β-fructosylnystose (GF4), which are formed when 2, 3 and 4 fructose units respectively are bound to one glucose unit by β-2,1 glycosidic linkages [3]. The growing demand for nutraceuticals or functional foods has directed a lot of research attention to scFOS due to their functional properties and economic potential in the pharmaceutical and food industry. As prebiotics, scFOS selectively promotes the growth of *Bifidobacteria* and *Lactobacillus sp.* in the large intestines and inhibit the growth of harmful microorganisms in the gut of humans preventing colon cancer. As functional foods, they are non-cariogenic, reduce cholesterol, phospholipids and triglyceride levels in the blood, promotes gut adsorption of calcium and magnesium, and have low caloric value [4]–[7].

Results have shown that scFOS behaves as sucrose in many properties including solubility, crystal data, freezing and boiling points [8]. Individually, 1-kestose, nystose and 1^F-fructofuranosylnystose are 63%, 45% and 32% respectively as sweet as sucrose [9]. Collectively, scFOS are about one-third the sweetness of sucrose [10]. scFOS are preferable over sucrose for use in foods because of their functional and health benefits, while sucrose causes rapid glucose jumps in the blood stream, significant metabolic energy and calories.

Substituting sucrose for scFOS increases beneficial bulking effects [11]. Under the same conditions, scFOS are found to have a higher viscosity and thermal stability than sucrose for similar concentrations of both substances [12]. scFOS have also shown high stability within the pH range of 4.0 - 7.0 and can remain stable for over a year at refrigeration temperatures [12].

Inulooligosaccharides (IOS) are known to possess functional and physicochemical properties similar to that of scFOS [13]. The IOS usually contains inulotriose (F3), inulotetraose (F4), inulopentaose (F5), 1-kestose (GF2), nystose (GF3), and 1^F-fructofuranosylnystose (GF4), in varying proportions. The scFOS is synthesised by the enzymatic polymerization of sucrose while the IOS is produced by the selective and partial hydrolysis of inulin [2]. The production methods of these two short-chain fructose-containing oligosaccharides are discussed in subsequent sections. Table 2.1 shows some of the companies that commercially produce short-chain fructose-containing oligosaccharides from sucrose or inulin and their trade names.

Table 2.1: Commercially available food-grade short-chain fructose-containing oligosaccharides [2]

Substrate	**Manufacturer**	**Trade name**
Sucrose	Beghin-Meiji Industries, France	Actilight®, Profeed®
	Cheil Foods and Chemicals Inc., Korea	Oligo-Sugar®
	GTC Nutrition, USA	NutraFlora®
	Meiji Seika Kaisha Ltd., Japan	Meioligo®
	Victory Biology Engineering Co., Ltd., China	Beneshune™ P-type
Inulin	Beneo-Orafti, Belgium	Orafti®
	Cosucra Groupe Warcoing, Belgium	Fibrulose®
	Sensus, the Netherlands	Frutalose®
	Nutriagaves de Mexico S.A. de C.V., Mexico	OLIFRUCTINE-SP®

2.3 scFOS synthesis from sucrose

Fructosyltransferases (FTase) and β-fructofuranosidase (FFase) are enzymes that act on sucrose in transfructosylating manner to produce scFOS [14]. The β-fructofuranosidase enzymes for scFOS synthesis have two main origins: Those of plant origin include asparagus, sugar beet, onion and Jerusalem artichoke, while other sources are of bacterial and fungal including *Aspergillus sp*., *Aureobasidium sp*., *Arthrobacter sp*., and *Fusarium sp*. Mass production of scFOS relies greatly on the enzymes of fungal origin, with those derived from *Aspergillus sp*. displaying high efficiency and yields [8], [15]. The enzyme used in this work is a protein-engineered variant of an *Aspergillus japonicus* β-fructofuranosidase [16]. The scFOS synthesis reaction mechanism follows the sequence GF → GF2 → GF3 → GF4. A sufficiently high concentration of the preceding oligosaccharide is always required for the formation of its homologue with a supplement fructose unit [17].

Many β-fructofuranosidases have been purified and characterized and most reports have identified the optimum conditions for effective transfructosylating activity to be at pH values between 5 to 6.5 and temperature values between 40 ^{o}C to 60 ^{o}C [18]–[24]. Most β-fructofuranosidases do not catalyze transfructosylation, except if there is a high enough concentration of the sucrose substrate between 200 to 700 g/L [25].

The main challenge associated with the use of β-fructofuranosidase in isolation is that the activity is inhibited as the concentration of glucose produced in the reaction mixture increases [26]–[31]. As this enzyme is invertase-type, the invertase activity needs to be repressed by ensuring a high enough concentration of the substrate sucrose. Due to this occurrence, the fructooligosaccharides form only 55-60% of the total dry mass of the sugars produced in a typical batch reaction [8]. Further purification steps are applied to enhance the purity of the commercial scFOS produced. Attempts to alleviate this effect include:

- The deployment of other glucose utilization enzymes, such as glucose oxidase together with the fructosyltransferase enzyme [32].
- The continuous *in situ* removal of glucose from the reaction mixture using a nano-filter membrane, to maintain the activity of β-fructofuranosidase throughout the reaction period and thus enhance the conversion rate [29].

» The modification of the protein structure of the β-fructofuranosidase enzyme to make it less susceptible to inhibition by glucose. The engineered β-fructofuranosidase enzyme to be applied in this study showed improved specific activity, thermosability, and reduced glucose inhibition [16].

2.4 Immobilization of β-fructofuranosidase

Despite their numerous advantages, the high cost of production, isolation and purification of enzymes is a limiting factor accompanying their low thermal and pH stability, when isolated from their natural environment. Recovery of active enzymes in soluble form from a reaction mixture is another technological challenge. Therefore, there is the need to improve the properties of enzymes, if they are to be exploited for industrial application. One tool for such improvement is by enzyme immobilization [33]. Immobilization is the process of limiting the free mobility of an enzyme by using techniques that allow re-use or continuous use of the enzyme to satisfy technical and economic demands. Table 2.2 shows the advantages of immobilization and its associated challenges. Immobilization can be achieved by several methods ranging from covalent binding to physical entrapment [34]–[38].

Table 2.2: Advantages and challenges of enzyme immobilization

Advantages of enzyme immobilization	**Challenges of enzyme immobilization**
Immobilization mimics the natural occurrence of enzymes in living cells providing the appropriate microenvironment for stability and resistance to environmental changes such as extreme pH and temperatures compared to free enzymes [33], [39].	In most cases there is reduction in activity of the enzyme due to protein interactions during the immobilization process leading to the loss of active sites [38], [40].
The heterogeneous nature of immobilized enzymes enables the easy recovery and reuse of enzyme, leading to higher enzyme productivity in terms of kg of products/kg of enzyme [33], [34], [36], [38], [39].	Increased diffusional mass transfer limitations for the substrate that adversely affect the rate of reaction, which can be minimised by appropriate reactor designs and careful enzyme carrier selection [33], [41], [42].
Allows for facile separation of product reducing protein contamination of product and eliminates downstream purification techniques thereby saving cost [36].	Supplementary costs of enzyme carrier and additional reagents.
The convenient handling of immobilized enzymes allows for continuous operations, rapid termination of reactions and a wider choice of reactor design [36], [38].	
Enzyme immobilization promotes the use of enzymes in multi-enzyme cascade processes by limiting enzyme interaction which leads to inhibition or deactivation of active sites [36], [43], [44].	
Higher resistance to shear stress. Increased specific activity in some cases due to better availability of catalytic centres [45].	

2.5 Methods of immobilization

Numerous methods of immobilization are available from literature; each method has its own efficiency and complexity. More importantly, one immobilization technique cannot efficiently

immobilize all enzymes. Therefore, several factors have to be taken into consideration when selecting a suitable technique of immobilization for a particular enzyme under specific conditions. It is sometimes expensive and labour intensive to develop a method of immobilization [45]. Figure 2.1 displays the various immobilization techniques.

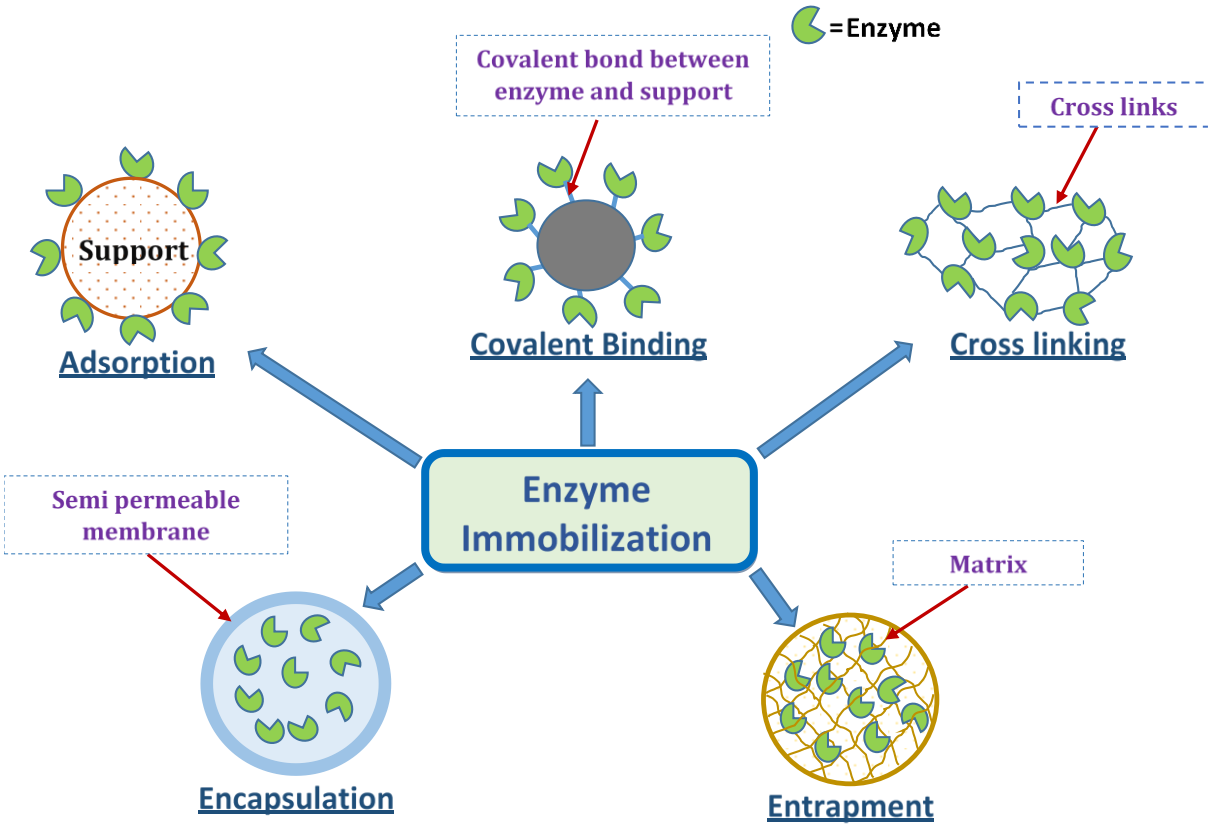

Figure 2.1: Different methods of enzyme immobilization

2.5.1 Adsorption

This method is among the simplest and comparatively the cheapest method of immobilization of enzymes [46]. Enzymes are bound to an insoluble matrix by hydrogen bonding, Van der Waals forces, hydrophobic interactions and/or ionic bonding [34], [38]. Enzymes are immobilized by simply mixing the enzyme solution with the suitable adsorbent under appropriate conditions of pH, temperature and ionic strength. Afterwards, washing out of any loosely bound or unbound protein is carried out [47].

These bindings are rather weak and typically do not alter the original structure of the enzyme, thus preventing the active sites from being disturbed and allowing the enzyme to retain its activity. Due to the weak nature of the bonds, the process can be reversed to regenerate the support material by altering the conditions that affect the strength of the interaction (pH, ionic strength, temperature and polarity of solvent).

This method seems to be economically attractive, but a major drawback is the desorption of enzyme molecules in solution, due to fluctuations in temperature or changes in substrate concentration. It is therefore advisable to maintain the initial reaction conditions throughout the reaction. Another major challenge of adsorption is low enzyme loading [48]. Aside from these disadvantages, adsorption remains frequently used in industrial applications especially in cases where the enzyme is not expensive. Commonly used adsorbents include: alumina, anion-exchange resins, calcium carbonate, carbon, cation-exchange resins and celluloses.

2.5.2 Entrapment

In this method, the enzyme is restricted to an environment where the substrate is able to penetrate but the enzyme cannot diffuse out. This can be carried out either in a polymer matrix (organic polymer or silica sol-gel) or in a membrane (microcapsule or hollow fibre) [36]. In this method, the synthesis of the polymer is carried out in the presence of the enzyme. Natural polymers used for entrapment include agar, agarose, gelatine, alginate and carrageenan. Synthetic polymers used include polyvinyl alcohol hydrogel and polyacrylamide [38], [42], [49]–[51].

There is no chemical modification of the enzyme, and therefore, the intrinsic properties of the enzyme are maintained. However, the gel formation process could sometimes result in enzyme deactivation and enzyme leakage. Entrapment is usually convenient with low molecular weight substrates and products; the difficulty lies with the inability of high molecular weight substrates to reach the active sites of the enzyme with ease [34], [36].
Membrane confinement can also be classified under this method. The only line of distinction is that the enzymes are confined by the pore sizes of a semi-permeable membrane. The membrane is able to retain the high molecular weight enzyme, while allowing the low

molecular weight compounds to diffuse through. Common membranes used include: nylon, cellulose, polysulfone and polyacrylate [29], [43], [52].

2.5.3 Microencapsulation

In this method, the enzyme is immobilized within a semi-permeable membrane microcapsule. This is carried out by an interfacial polymerization technique, by agitation of an organic solvent containing one component of a copolymer with surfactant in a vessel followed by the addition of aqueous enzyme solution [53]. The polymeric membrane forms at the liquid-liquid interface with the aqueous phase dispersed as small droplets [53]. Enzymes are restricted by the membrane wall, but free-floating in the core space. The limited access to the interior of the microcapsule protects the enzyme from harsh environmental conditions, hence improving the stability [54]. Immobilized enzymes produced by this technique provide a large surface area. However, this technique is not appropriate for high molecular weight substrates due to the relatively smaller pore size [35], [55].

2.5.4 Covalent binding

This technique is most investigated in recent times. Immobilization is achieved by the formation of covalent bonds between functional groups on the enzyme and the support matrix. Functional groups involved in the covalent bond formation are usually those that are non-essential for the catalytic activity of the enzyme. The functional groups of proteins appropriate for covalent binding under mild conditions are either nucleophilic or electrophilic. The electrophilic functional groups include:

- The alpha amino groups of the chain and the epsilon amino groups of lysine and arginine.
- The phenol ring of tyrosine.
- The thiol group of cysteine.
- The hydroxyl groups of serine and threonine.
- The imidazole group of histidine.
- The indole group of tryptophan [37].

The nucleophilic functional groups include:

- The alpha carboxyl group of the chain end and the beta and gamma carboxyl groups of aspartic and glutamic acids [56].

In some cases, the functional groups on the support material are activated with certain reagents before the enzymes can be bound to the support covalently. The most commonly used coupling reagents are carbodiimide and glutaraldehyde [57]–[59]. This method minimises leaching of enzyme, due to the strong nature of the covalent bonds. This also renders the enzyme very stable compared to other techniques of immobilization, hence the most convenient method if enhancing stability of the enzyme is the paramount purpose of immobilization [60].

A major drawback is the high cost of immobilization, due to the irreversible nature of the process, since the covalent bonds formed cannot be broken easily and therefore the support material cannot be regenerated. Also, there could be losses in enzyme activity should the amino acid residues in the active sites of the enzyme be involved in the binding process. A process that sometimes improves the activity yield, is to conduct the coupling reaction in the presence of substrate analogues to prevent the active site residues from taking part in the binding process [34]. This technique can be applied even if the properties of the enzyme in question are not explicitly known, due to the availability of a wide range of insoluble carriers with functional groups capable of covalent coupling or being activated to give such groups.

2.5.5 Cross-linking

This method involves the binding of enzymes molecules to each other by the use of bifunctional or multifunctional reagents like glutaraldehyde. This results in the formation of very high molecular weight aggregates. No support material is required in this technique; enzymes are bound to each other by covalent bonds. There is always a high tendency of alterations in the conformation of the active centres of cross-linked enzymes, since cross-linking reactions are performed under relatively harsh conditions; this may lead to an appreciable loss of activity. [42].

The advantages of this technique include:

- It may be less expensive since a potentially expensive support material is not used.
- Reduction in mass transfer limitations due to the absence of a bulky carrier material.
- Improved pH and thermal stability of the enzyme [61].
- Concentration of activity in enzyme compared to carrier bound enzymes [62].

The major disadvantages include:

- Low activity retention due to involvement of active sites in the chemical binding and the use of some enzyme molecules as support [62].
- Low mechanical stability and difficulty in handling the gelatinous enzyme aggregates.
- The toxicity of most bifunctional reagents [63].

As a way to circumvent these drawbacks, this method is normally used in conjunction with support bound techniques of immobilization to enhance enzyme properties [64].

Generally, the binding strength of an immobilization method is inversely proportional to the ease of reversibility of the method. These two contradictory requirements, i.e. stability and reversibility, are seemingly impossible to fulfil simultaneously in a single immobilization technique. The traditional approach focuses on improving binding strength at the expense of reversibility. Table 2.3 provides a summary of the pros and cons of the various immobilization techniques.

Table 2.3: A summary of the various immobilization techniques

Characteristic	Adsorption	Covalent binding	Entrapment	Encapsulation
Preparation	Simple	Difficult	Difficult	Simple
Cost	Low	High	Moderate	High
Binding force	Variable	Strong	Weak	Strong
Enzyme leakage	Yes	No	Yes	No
Applicability	Wide	Selective	Wide	Very wide
Running Problems	High	Low	High	High
Matrix effects	Yes	Yes	Yes	No
Large diffusion barriers	No	No	Yes	Yes
Microbial protection	No	No	Yes	Yes

2.6 Immobilization parameters

It is very important to know the extent of or how successful an immobilization technique was, in terms of how much enzyme is immobilized as well as how much activity is retained. This knowledge provides grounds for comparison and ultimately aids in making informed decisions as far as immobilizing a particular type of enzyme is concerned. A few of such measurable parameters are discussed below. It should be noted that there are slight differences as to how several authors have defined these parameters [65].

2.6.1 Immobilization yield

The immobilization yield (Y_i) is a collective measure of the amount of enzyme as well the fraction activity of the starting enzyme solution that is taken up by the support material during the immobilization process [65]. A high immobilization yield is desirable since it indicates the suitability of the particular immobilization technique for the enzyme of interest. The constraint of this parameter is that it fails to measure the activity loss as a result of the mass transfer limitation of substrate in and out of the carrier matrix. It also fails to show the activity loss as a result of change in protein conformation, restriction of enzyme mobility or loss of active sites.

Immobilization yield is estimated using equation {1}.

$$Y_i = \frac{(A_i - A_f)}{A_i} \, x \, 100 \qquad \{1\}$$

Where A_i (total initial enzyme activity) is the total number of activity units of the starting enzyme solution used for the immobilization process.

A_f is the number of activity units found in the filtrates and washing solutions after immobilization. In some instances, protein concentrations are used to determine the immobilization yield, but it is not accurate and can be deceptive in case of impure enzymes. It is however appropriate to estimate the immobilization yield based on the protein concentration and enzyme activity, in order to better understand the enzyme or protein loading onto the carrier material [65].

2.6.2 Immobilization efficiency

Immobilization efficiency, (E_f) also referred to as activity yield [65], [66], is a measure of the fraction of the immobilized activity that is actually reflected by the enzyme bound to the

carrier material. This parameter exposes the fraction of activity lost as a result of the combined effect of mass transfer limitation, change in protein conformation and loss of active sites. But it is unable to differentiate the inherent loss of activity as a result of deactivation or conformational changes from the apparent loss of activity as a result of mass transfer limitation or unavailability of some active sites to substrates. A high activity yield also indicates that the given immobilization technique is appropriate for the particular enzyme being immobilized.

It is calculated using equation {2} below.

$$\mathrm{E}_f = \frac{A_m}{(A_i - A_f)}\, x\, 100 \qquad \{2\}$$

Where A_m is the number of activity units on the support material after immobilization and washing.

It is possible to obtain an immobilization yield of 100%, but an immobilization efficiency of 0% is indicative of the fact that all the bound enzyme has been deactivated on the carrier material possibly as a result of blocking of active sites or conformational changes to the protein structure.

2.6.3 Activity recovery

The activity recovery (A_r) is a measure of the overall success of the immobilization process. It estimates the amount of activity of the starting enzyme solution that is actually reflected on the immobilized enzyme [65].

$$\mathrm{A}_r = \frac{A_m}{A_i}\, x\, 100 \qquad \{3\}$$

It should be noted that all the activity assays for the estimation of all these parameters need to be carried out under the exact same conditions.

2.7 Choice of support for enzyme immobilization

The major target of most experimental immobilization investigations is to deploy an insoluble enzyme support that enhances stability and facilitates recovery of enzyme, thus allowing for repeated use of enzyme or design of continuous systems. There is no universal support material for enzyme immobilization. Therefore, the choice of appropriate support for an enzyme is very important in the quest to retain high activity and enhanced stability under conditions that are economically viable, since the requirements are specific for every enzyme.

A good knowledge of both the chemical and physical properties of the carrier is essential to make informed decisions on the most suitable support for the enzyme in question. Even with that, one cannot simply predict which support would be most appropriate for an enzyme. The main chemical properties which affect enzyme binding capacity include:

- Chemical composition of the support material
- Chemical stability under specified reaction conditions
- Types of functional groups available for interaction with enzyme

Whereas the physical properties essential for good support materials include:

- Appropriate Surface area
- Hydrophilic character
- Thermal stability
- Suitable particle size and shape
- Permeability for substrates and products
- Insolubility in reaction media
- Resistance to microbial attack [34]
- Swelling behaviour
- Accessible volume of matrix
- Flow resistance in case of fixed-bed applications

Another highly relevant factor to consider is the overall cost of the support material in the industrial process. It should be either cheap enough to discard or be able to be regenerated after the useful lifespan of the immobilized enzyme. Any support material selected for immobilization of enzyme should possess a good number of the aforementioned desirable qualities.

Certain support matrices possess some peculiar properties that give them an advantage over others. Some of these properties include:

- Magnetism that enhances transfer, stabilization and separation of enzymes by applying of a magnetic field [60], [63], [67]–[70].
- Nano particles and fibres have large surface areas for high enzyme loading [71], [72].
- Nonporous supports may eliminate diffusion constraints.
- Membrane surfaces for continuous operation, short residence time and low internal and external resistance [73], [74].

2.8 Some supports used in β-fructofuranosidase immobilization

2.8.1 Chitosan

Chitosan is a natural polysaccharide obtained from chitin. Chitin is an abundant renewable natural resource found in shells of crustaceans, the exoskeletons of insects and the cell walls of fungi, where it provides firmness and strength. It is obtained commercially from low cost shells of shellfish and wastes of the seafood processing industry [33]. Chitin is a long chain polymer composed of β(1-4)linked 2-acetamido-2-deoxy-β-D-glucose units (or N-acetyl-D-glucosamine).

Chitosan is a copolymer of N-acetyl-D-glucosamine and D-glucosamine. Chitosan has reactive amino and hydroxyl groups, which are susceptible to chemical modifications. Chitosan offers quite a number of unique characteristics, which make it one of the most promising natural polymeric carriers for enzyme immobilization. These characteristics include biocompatibility, biodegradability to harmless products, nontoxicity, physiological inertness, antibacterial properties, heavy metal ion chelation, gel forming properties and hydrophilicity [33]. Glutaraldehyde is usually used as the cross-linking and activating agent for immobilization of enzymes on chitosan supports. This is due to the presence of amino groups on both enzyme and chitosan surfaces [75]. Since scFOS is a food grade product, the use of poisonous coupling agents like glutaraldehyde is disadvantageous [39], [42].

2.8.2 Alginate beads

Alginate is a natural heteropolymer obtained from brown algae in the form of calcium, magnesium, strontium and sodium salts of alginic acid [76]. This polymer is made of 1-4 linked β-D-mannuronic (M) and α-L-guluronic acid (G) monomers in varied compositions depending on the source. The arrangement is such that there are homopolymeric regions of MM and GG blocks with alternating heteroplymeric MG blocks intermingled among them [77]. The guluronic blocks possess active sites with high affinity for most divalent cations except Mg^{2+} [78], These interactions are responsible for the formation of the alginate gel [77]. Hence, the strength of the alginate gel is highly dependent on the G content-High G content indicates high gel strength. When a solution of sodium alginate comes into contact with a solution containing calcium, there is instant precipitation of calcium alginate at the interfacial region

followed by a more gradual gelation of the interior as the calcium ions diffuse through the alginate. The formed gel has:

- **Mechanical stability and strength** making the gel resistant to shear forces and also providing the option of being packed into a column without collapsing.
- **Biochemical inertness,** which is an essential characteristic of a potential catalyst carrier since it is not capable of causing any chemical or biological changes to cells or enzymes as well as not causing any interference during reactions.
- **Large number of interstitial pores and spaces** making it conducive for immobilizing cells and enzymes and allowing for rapid diffusion of products and substrates in and out of matrix pores.

Pb^{2+} and Cu^{2+} usually form stronger and stable gels due to their high affinity for alginate. However, they cannot be used in food applications because of concerns about their toxicity. In as much as alginate appears as a very convenient carrier for enzyme immobilization, there are few limitations associated with it.

- **Protein leakage:** The large interstitial spaces that allow for rapid diffusion of substrate and product also cause the leakage of immobilized cells and enzymes out of the gel beads. Protein leakage from alginate beads can be minimized by cross-linking enzyme with glutaraldehyde prior to the entrapment process. They can also be bound to activated charcoal prior to the entrapment but because of the toxicity of many of these cross-linking agents, their use in food application is unadvisable. Beads of high alginate density could also be used [77], [79].
- **Low volumetric activity:** The usually low mass of immobilized enzyme/cell per mass of beads consequently leads to low volumetric activity, since a larger reactor volume is required to accommodate the low amount of immobilized catalyst due to the bulky nature of the alginate gel [79].
- **Susceptibility to microbial contamination:** The high amounts of carbon and water in the gel increases the likelihood of getting contaminated even when stored at low temperatures [79].

2.8.3 Ion exchange resins

Enzymes are immobilized on ion exchange resins by either adsorption or by ionic binding. The use of ion exchange resins seems to be a reasonable choice, to alleviate the cost of immobilization by providing the option of regeneration and re-use of the enzyme support. However, the main challenge is the leaching of enzyme during abrupt changes in temperature and pressure.

Highly porous strong base anion exchange resins have produced very good adsorption results in the immobilization of β-fructofuranosidase and are usually preferred over the other types of resins [80], [81]. This is because the enzyme has an isoelectric point (pI) between pH of 4 to 5 [19], [82], [83]. Hence it possesses a negative charge and is strongly attracted to anion exchange resins. To obtain good results, the adsorption process has to be carried out at a pH greater than the pI of the β-fructofuranosidase enzyme [80].

2.8.4 Ceramic membrane

Membranes generally provide high surface area per unit volume and also the possibility to control the composition of the reaction mixture. Not only are membranes used for separation of enzymes from reaction mixtures, they can also provide a surface for enzyme immobilization [84]. Usually, enzymes are immobilized onto membranes by confinement (entrapment) in the membrane pores. However, other techniques can also be used such as: covalent binding, cross-linking and adsorption [85].

2.8.5 Porous glass and silica

Mesoporous silicates provide large surface area, narrow pore size distribution, well-defined pore geometry, high thermal and mechanical stability, and toxicological safety [39]. They are also amendable to chemical surface modification with various functional groups that strengthen enzyme and support bonds [76]. These properties make silica a good candidate for enzyme immobilization. Silicates are applicable in all the various carrier bound immobilization techniques depending on the modification of the silica support.

2.8.6 Functionalized magnetic particles

Magnetic microparticles or nanoparticles normally have a magnetite (Fe_3O_4) core embedded in an organic or polymeric shell [86]. Enzymes are generally immobilized onto these particles

by covalent binding or by cross-linking. Glutaraldehyde and derivatives of carbodiimide are generally used as the binding reagents, depending on the target functional groups. The main advantage of these carriers is the ease of separation (by applying a magnetic field) and process control. These particles are basically non-porous and have high surface area to volume ratio, making them preferable compared to porous macro carriers. The disadvantage of using these particles is the activity loss during the covalent binding process and the use of poisonous cross-linking chemicals. Also, the particles cannot be regenerated implying a high cost of immobilization [63].

2.8.7 Methacrylamide-based polymeric beads

These are synthetic polymer beads formed from the copolymerization of methacrylamide and glycidyl methacrylate (a monomer carrying oxirane group). It can be classified among the epoxy-activated acrylic support used for immobilization of numerous enzymes including transferases [37]. They are highly hydrophilic and both chemically and mechanically stable [36]. Generally, enzymes are immobilized onto these supports covalently by reactions between the oxirane moieties and the free amino groups on the enzyme under alkaline or neutral conditions. The immobilized enzymes have good stability and enhanced activity. The epoxy functional groups also offer a lot of benefits including:

- Single step binding of enzyme
- Short spacer arms and ability to react with several nucleophilic groups on the enzyme molecule.
- High stability

Table 2.4 displays some published works on scFOS production using enzymes immobilized on various carriers.

Table 2.4: Published works on some carriers used to immobilize enzymes for scFOS synthesis

Method	Carrier	% protein immobilized[a]	(Y_i)	(E_f)	% scFOS[b]	Reference
Adsorption	DOWEX-1X8-50	93	*	*	*	[80]
	WA-30	86	*	40	51.9	[3]
	Dowex Marathon MSA	*	65	11.1	*	[87]
	Amberlite IRA 900	*	*	13.9	*	[87]
Entrapment	Calcium alginate	66	60	*	*	[88]
	Dried alginate-entrapped enzymes (DALGEEs)	47	50	*	46	[79]
Covalent binding	Eupergit C	*	*	96	57	[89]
	Chitosan-coated magnetic nanoparticles	*	*	80	59.5	[63]
	methacrylamide-based polymeric beads	*	100	*	60	[90]
Simultaneous cross linking and covalent binding	Chitosan beads	*	81	93	72.2	[17]
Simultaneous cross linking and entrapment	Calcium alginate	*	90	52	68.5	[17]

a % (total amount of protein in solutions after immobilization/amount of protein in solution before immobilization)
b % (g of scFOS/g of sucrose); ***** Value not reported in literature

2.9 Optimal conditions for the free and immobilized β-fructofuranosidase

The β-fructofuranosidase enzymes immobilized on various supports, have been extensively studied and compared with the free enzyme counterpart in terms of optimal pH and temperature, pH, temperature and storage stability and scFOS yield. The optimal pH and temperature have been found in most cases to be the same for both the immobilized and free enzymes (shown in Table 2.5). However, the immobilized enzyme usually has greater stability over a wider range of pH and temperature as expected. The immobilized enzyme maintains

its activity for a longer period of storage compared to the free enzyme. The free enzyme usually has the upper hand regarding the yield of scFOS produced.

Table 2.5: Some published works on the optimal conditions for some free and immobilized β-fructofuranosidase enzymes.

Source of enzyme	Immobilized		Free		Reference
	pH	Temp, °C	pH	Temp, °C	
A. aculeatus	6.5	66	6.5	66	[91]
A. japonicus	5.4	60	4.0-5.5	60-65	[51]
A. japonicus	5.5	60	5.5	60	[75]
A. japonicus	5.5	60	5.5	60	[63]
A. flavu	5.5-7	40, 60	5.5	50	[92]
S. sclerotiorum	4.5, 5.5	55, 60	5	60	[93]
Pectinex Ultra SP-L	5.5-6.5	65	5.5-6.5	65	[89]
A. niger	5	60	5	60	[90]
A. awamori	5	60	5	60	[66]
A. pullulans	5.5	65	5.5	60	[47]
A. japonicus	5.4	60	5.4	60	[90]

2.10 Commercial scFOS production from sucrose

Commercial production of scFOS from sucrose can be classified in two main categories namely: the production of scFOS using free enzyme and the production of scFOS using immobilized enzymes or cells [29], [52]. Using a conventional batch soluble enzyme system, scFOS is produced commercially by mixing the biocatalyst with 50 - 60% (w/v) sucrose solution at pH 5.5 - 6.0, and temperature of 50 - 60 °C with continuous mixing for 4 - 20 hours. To stop the enzymatic activity, the reaction mixture (mostly made up of scFOS, fructose, glucose and unconverted sucrose) is heated to 90 °C for a period of 30 minutes followed by cooling below 50 °C and a series of purification steps [29]. The major drawback of this system is the high cost of using a fresh biocatalyst per reaction. The immobilized enzyme/cell system follows the same sequence, except for the biocatalyst deactivation step. Rather the reaction mixture is

separated from the immobilized catalyst using the appropriate technique applicable to the type of reactor being used.

The high concentration of the sucrose substrate alleviates the cost of evaporation during concentration of product. Also an enzyme with high transfructosylating ability is recommended [94]. Vankova et al. (2008) reported a design for industrial production of 10 000 tonnes of scFOS per annum using fructosyltransferase (FTase) from *A. pullulans* immobilized on ion exchange resins. Removal of glucose, fructose and unreacted sucrose was carried out using a simulated moving-bed chromatography to obtain high contents of scFOS [95].

2.11 IOS production by hydrolysis of inulin

IOS is produced by the controlled enzymatic hydrolysis of inulin using inulinase. While exo-inulinase hydrolyses inulin by cleaving fructose from the non-reducing end of inulin resulting in ultra-high fructose syrup, endo-inulinase hydrolyses inulin to form a mixture of inulooligosaccharides of varying degrees of polymerization. Inulooligosaccharides production therefore relies on the application of endo-inulinase [5], [96], [97]. The endo-inulinase acts on inulin to break down the β-D-(2→1) glycosidic linkages randomly and produces a mixture of inulooligosaccharides containing β-D-Fru(1→2)-[β-D-Fru(1→2)-]$_n$, where n = 1 - 9 and α-D-Glu(1→2)-[β-D-Fru(1→2)-]$_n$, where n = 2 - 9 [4]. The major sources of inulinase include *Aspergillus* spp., *Penicillium* spp., *Bacillus* spp. and *Pseudomonas* spp. [98]. Reports have placed the optimal hydrolysis temperature and pH ranges between 37 - 60 ^{o}C and 5.0 - 7.0 respectively [99], [100]. Under these conditions, higher IOS yields (60 to 86%, based on inulin dry mass) have been obtained compared to that of scFOS from sucrose. A maximum inulin conversion of 92% was reported when pure inulin was applied at 50 ^{o}C and pH 5.8 [101]. A yield of 89% was achieved when IOS was produced using endoinulinase from *A. ficcum* at 50 ^{o}C, pH 5.0 and 72 h [102].

2.12 Sources of inulin

Interests in inulin and inulin containing crops especially in Europe has increased due to the wide range of potential applications [103]. While inulin is present in many plant species, only in Jerusalem artichoke, chicory, agave and dahlia does it accumulate in sufficient quantities.

Furthermore, Jerusalem artichoke and chicory have inulin in quantities adequate (>15% on wet weight basis and >65% on dry weight basis) for exploitation as viable agricultural sources for inulin [104]. Commercial production of IOS from inulin over the years has relied mainly on chicory rather than Jerusalem artichoke, mainly because of the applicability of sugar beet extraction machinery to chicory processing. Another probable advantage of chicory is that around 71% of its inulin has degree of polymerization ≥ 9 as compared to 48% in Jerusalem artichoke [103]. This gives a wider range of application for the chicory inulin. Due to the increasing demand for inulin, additional sources are under investigation of which Jerusalem artichoke is the prime candidate [105]. The potential of Jerusalem artichoke is discussed at length in the subsequent sections.

2.13 Jerusalem artichoke

Jerusalem artichoke (*Helianthus tuberosus* L.), also referred to as sunchoke, sunroot, topinambur or woodland sunflower is a perennial herbaceous plant in the same *Asteraceae* family as sunflower (*Helianthus annuus* L.). It originates in North America and is usually cultivated as an annual crop [103]. The versatile ability of Jerusalem artichoke to thrive under harsh soil and climatic conditions is demonstrated in the successful cultivations in Northern Europe, China, Korea, Australia and New Zealand [106]. Unlike most agricultural crops, Jerusalem artichoke (JA), has a high growth rate even in poor soils with little or no irrigation and fertilizer requirements, good tolerance to frost and drought and strong resistance to pests and diseases [107]–[109]. Therefore, cultivation of JA does not compete with food crops for arable land. Rather, its cultivation improves salt-alkaline, oil-polluted and coal-mining soils [105]. Comparing the agronomic performances of chicory and Jerusalem artichoke in Germany, it was observed that the inulin yield losses as a result of weed competition in Jerusalem artichoke was much lower (8%) compared to that of chicory (47%).

Jerusalem artichoke aerial biomass dry matter yields of 18.1 - 31.1 tonnes per hectare (t/ha) and dry weight tuber yields of 9.1 - 10.6 t/ha have been reported [110]. In a survey by Gunnarsson et al. (2014) in Sweden, after planting 11 Jerusalem artichoke clones in mid-May, the mean dry matter content of the aerial biomass increased from 20% to 61% over the harvesting period of September to December. A similar trend was also noticed for the tuber (the highest fresh tuber yield of 4.4 kg/m^2 was obtained in the December harvest) [111].

Harvesting time therefore plays a crucial role in the biomass density. The roots grow rapidly, reaching a mass 25 kg per plant at the end of 24 weeks, beyond which they begin to decline. The tubers constitute a major fraction of the biomass of JA. The tubers come in various shapes and sizes (length to diameter ratio) depending on the age, planting conditions and the JA clone [112]. The tubers usually consist of about 80% water, 15% carbohydrates and 1-2% protein.

1.3.1 The utilization of Jerusalem artichoke in a biorefinery

A biorefinery provides the principal opportunity to convert almost any type of biomass into different types of products, by the combination of suitable biotechnological and chemical techniques [107]. It is also paramount to consider sustainability in terms of economics, resource application and social impact. Therefore, selection of the appropriate biomass for the application in a biorefinery is an essential factor. Most available literature on biorefinery research is focused on forest biomass, algal biomass, agricultural and /or food waste, and crops cultivated on marginal land [113]. Literature on the cultivation of crops for biorefinery applications is rather scarce, the most likely reason being the ongoing debates regarding the use of agricultural land for fuel production, while starvation and malnutrition still exist [114]. For a biorefinery using cultivated crops to be competitive, it should be able to produce at least one product of high value, preferably a food product, coupled with energy product(s) from residues. One approach for producing such chemicals is by depolymerisation and/or fermentation of biopolymers [115].

Jerusalem artichoke comes across as a very suitable plant for biorefinery application, due to its ability to thrive well on non-fertile lands with relatively minimal cultivation expenses thereby obviating the issue of competition with agricultural/food crops for arable land [112]. Its ability to produce large amounts of biomass in short time intervals also alleviates the challenge of consistent supply of feedstock [103]. The Jerusalem artichoke biomass contains a wide variety of chemicals and polymers that are precursors for production of high value products. The carbohydrates content (mostly inulin), when hydrolysed and fermented, can yield ethanol, L-lactic acid, acetone, butanol, 2,3-butandiol, butyric acid, succinic acid, sorbitol or biodiesel, based on the microorganism and conditions applied [107]. The inulin when subjected to appropriate enzymatic hydrolysis, it yields IOS for application as a functional

food. Proteins when extracted or hydrolysed into amino acids find application mainly in the food industry due to their nutritional and functional properties [103], [111]. Anaerobic digestion of inulin and cellulosic residues also yields biogas for fuel [116].

2.14 Major components of Jerusalem Artichoke

2.14.1 Inulin

Unlike most crops that store carbon as starch (a polymer of glucose), Jerusalem artichoke stores carbon as inulin (a fructose polymer) [103]. Inulin is a linear biopolymer of D-fructose units connected by β (2,1) glycosidic linkages, and terminated with one D-glucose molecule linked to the fructose chain by an α (2,1) bond. This plant-derived polysaccharide resembles starch, appears as a white powder with a neutral taste and is the main carbohydrate of Jerusalem artichoke [117]. Inulin constitutes about 15 to 30% of fresh weight and about 65 to 80% of dry weight of the JA tubers. [111], [112]. This composition is comparable to that of chicory (inulin content is 70-80% of dry weight) [103]. The degree of polymerization (DP) of the inulin varies widely between DP of 2 and 70, based on factors such as the species, cultivar, production conditions and the physiological age of the JA tuber. Based on the degree of polymerization, the inulin can be classified as IOS (DP ≤ 5), native inulin (DP 10-12) or High-Performance inulin (HP-inulin) (DP >12). The IOS are usually desirable for their numerous prebiotic and health applications. The native inulin and the HP-inulin are less soluble and find application as fat replacement in dairy products, table spreads, baked goods and frozen desserts [118].

2.14.2 Inulin extraction

The solubility of inulin in water is directly proportional to temperature. From almost zero solubility at 25 ^{o}C, the solubility increases to 35% (w/v) at 90 ^{o}C. Industrial inulin extraction therefore relies greatly on hot water diffusion [119]. Most extraction methods in literature make use of the hot water as solvent with slight adjustments in temperature, extraction time and solid loading to obtain maximum inulin yields. As a pretreatment step the JA tubers are sliced or milled into minute particles to facilitate the rate of diffusion. In a report by Yang et al. (2015), optimal extraction condition of 70 ^{o}C extraction temperature, 15:1 water to Jerusalem artichoke ratio, 90 min extraction time and two cycle of re-extraction produced an inulin yield of 89.5% [107]. Some enzymatic methods of inulin extraction have been

attempted. However, the yields obtained were of not significantly different from the hot water methods. It was therefore established that some mechanical means was required [120]. Others have also recorded slight improvements in inulin yields (from 10.8% to 12.2% on wet weight basis) when sonication and microwave-assisted means of extraction were deployed as against the conventional hot water method [121], [122]. These other methods have only gained credence on the lab scale.

2.14.3 Proteins

The content of proteins in the Jerusalem artichoke tubers is within the range of 5.3% to 15% of dry weight [103]. The various proteins found in Jerusalem artichoke tubers have not been fully characterized. However, content of amino acids in Jerusalem artichoke tubers is sufficient in favourable proportions compared to chicory and potatoes, especially the essential amino acids and the sulphur containing amino acids [115]. The JA tuber is rich in lysine and methionine considered of high quality for food and feed applications [103]. The JA tuber proteins also find application as nutraceuticals and ingredients of functional foods [123]. Besides the conventional application of proteins directly in the food industry, proteins are also a potential source of platform chemicals such as acrylamide, benzoic acid and 1,2-ethanediamine, which can be obtained by firstly degrading the proteins into amino acids from which such platform chemicals can be produced [124]. Plant proteins have also been applied industrially in manufacturing biobased and biodegradable plastics. The sulphur containing proteins exhibit good foaming abilities and hence could also be applied in production of insulation materials [125].

2.14.4 Protein extraction

Protein extraction is usually carried out in three steps namely: solubilisation, precipitation and recovery [108].

2.14.4.1 Alkaline solubilisation of proteins

Protein solubilisation using alkali solutions especially NaOH, is an industrially accepted practice for plant-based proteins from canola, peas and soybean. The alkali solution interrupts the protein-protein bonds, thereby liberating the proteins in the aqueous medium. The solubility of the proteins is highly dependent on the pH, temperature and concentration. The pH is usually varied between 7.5 and 13.0 depending on the source and nature of proteins to

be extracted. High protein solubility and consequently high protein yield is obtained at high pH (>12), but the high negative charges could cause proteins to denature [126]. At pH >13 alkaline hydrolysis of peptide bonds occurs, leading to rampant cleavage of the protein molecules. In a report, pH 9.0 and 25 ^{o}C were revealed as the optimal conditions for the extraction of soy protein isolates (88.1% w/w dry basis) [127]. The use of sodium hexametaphosphate (SHMP) as an alternative was explored. The proteins isolated exhibited better taste and colour, although lower protein yields were obtained with SHMP compared to NaOH, which is possibly the reason for its discontinued use [128]. There is yet to be an extensive data in literature on the extraction protocols, conditions and protein yields regarding the extraction of proteins from Jerusalem artichoke. This could probably be due to the sporadic interests in the plant over the years. However, an earlier report revealed a protein isolate extracted from Jerusalem artichoke forage contained 67 to 76% (w/w dry mass) protein; protein isolate yield of 800 kg/ha from the forage was also estimated [108].

2.14.4.2 Precipitation of solubilized protein with dilute acids

A centrifugation or filtration process usually precede this step to separate the biomass residues from the solubilized protein to improve the purity of the protein obtained in the end. The precipitation step relies on the fact that proteins demonstrate minimum solubility at their isoelectric point (pI) [128]. By manipulating the solubility of the protein in tandem with filtration techniques, protein concentrates and isolates of variable purity and functionality can be obtained. The pH of the alkali protein solution is adjusted by addition of dilute HCl or acetic acid until to obtain a pH≈pI at which precipitation of the protein occurs. Most proteins have their pIs within the range of pH 4 to 8 therefore, the optimum precipitation pH (for optimum protein yield) varies with the protein of interest [53].

2.14.5 Recovery of proteins

The protein recovery step is very critical in minimizing the amounts of antinutritional factors (examples include glucosinolates, phenolics and phytates) in the protein isolates [129]. These antinutritional factors, when present in large quantities in a protein isolate, introduce inferior physicochemical properties, poor digestibility, objectionable colour and bad taste to the protein [128]. The recovery is usually carried out by ultrafiltration. Yellow pea proteins exhibit excellent physicochemical properties when recovered by ultrafiltration [130]. In some

instances, ultrafiltration is coupled with diafiltration to further improve the purity of the protein. The ultrafiltration/diafiltration process has been reported to generate concentrated protein isolates with 69.1 – 88.6% (w/w dry weight basis) protein content [131].

2.14.6 Other carbohydrates

Besides inulin, other carbohydrates that exist in Jerusalem artichoke include sugars (predominantly fructose and glucose along with sucrose, xylose, galactose, mannose, arabinose and rhamnose in minute quantities), cellulose and hemicellulose. The fructose and glucose content of Jerusalem artichoke tubers is about 4 - 5% of the dry weight [115]. Cellulose and hemicellulose make up a greater portion of the aerial biomass. Dry matter yields of 15 – 25% and 11 – 13% of cellulose and hemicellulose content in the JA biomass have been reported [111]. The complexity of these chemicals and the high lignin content (17 – 19% dry weight) in the stalks have restricted their usefulness [132].

2.15 Ethanol production from Jerusalem artichoke tuber residues

An appreciable amount of residual inulin is usually trapped in the Jerusalem artichoke fibres after the inulin extraction process, which renders an average inulin yield of 80% [99]. While the residues (mainly unrecovered inulin, sugars, cellulose and hemicellulose) can be used as cheap animal feed or combusted to produce heat or power, the question about sustainability and economic viability then arises with respect to the biorefinery use of Jerusalem artichoke. An unexplored option is the valorisation of these inulin-rich residues through fermentation to produce bioethanol by using enzymes and microorganisms. While there is virtually no data on ethanol production from the residual inulin in the JA tuber residues after inulin/IOS and protein extraction, the pre-treatment and fermentation processes could be identical to the prioritized production of ethanol from JA inulin. The two routes for bioethanol production from Jerusalem artichoke inulin are discussed below [111].

2.15.1 Separate hydrolysis and fermentation (SHF)

The separate hydrolysis and fermentation (SHF) method is characterized as inulin hydrolysis and sugar fermentation being conducted in two separate process steps. Typically, inulin in the JA tuber is by hydrolysing into fermentable sugars (fructose and glucose), using either dilute mineral acids or hydrolytic enzymes. Subsequently, the fermentable sugars are fermented

into ethanol employing yeasts such as *Zymomonas mobilis*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* [107].

For the ethanol production from JA inulin by the SHF process, the hydrolysis step has great influence on the following fermentation step. Complete hydrolysis of inulin produces maximum amount of sugar and consequently high ethanol yield. However, the hydrolysis process using mineral acids may generate some by-products which inhibit the activity of the yeast in the fermentation step, thereby prolonging fermentation time. The effect of acid or enzymatic hydrolysis of inulin on the consequent alcoholic fermentation was investigated by Toran-Diaz et al. (1985) [133]. It was discovered that acid hydrolysis was more rapid than enzymatic hydrolysis, but the by-products from acid hydrolysis inhibited the growth of yeast in the following fermentation step, resulting in minimal ethanol yield.

Razmovski et al. (2011) investigated the influence of temperature and residence time on acid hydrolysis of inulin from JA [134]. The Jerusalem artichoke hydrolysates obtained under the varied hydrolysis conditions were evaluated further in ethanol fermentation with *S. cerevisiae* as a biocatalyst. It was discovered that acid hydrolysis at elevated temperatures and prolonged residence times increased the concentration of the yeast inhibitor hydroxymethylfurfural (HMF) and accelerated the degradation of sugars. Kim and Hamdy (1986) suggested 0.1M HCl at 97 ^{o}C and 15 minutes as the optimal conditions for hydrolysis of JA tuber slurry to obtain complete hydrolysis with reduced yeast inhibitor generation [135].

In another report, several enzymes were tested for the hydrolysis of inulin in JA tubers. Results indicated that cellulolytic enzymes such as Novozym 188 and Calluclast are very poor at hydrolysing JA inulin by the very low amounts of glucose and fructose they produced [107]. On the other hand, Novo 230 was pointed out to be the most efficient enzyme for inulin hydrolysis under the authors' experimental conditions. It must be noted that more recent data on ethanol production from JA tubers by the SHF process is not readily available possibly due to the better prospects of ethanol production by Simultaneous saccharification and fermentation (SSF).

2.15.2 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) is characterized as inulin hydrolysis and sugar fermentation being performed simultaneously in a one process step (one bioreactor) using combination of biocatalysts (enzymes and micro-organism); acid hydrolysis is not an option for an SSF process. Such a direct conversion of inulin into ethanol is advantageous from capital investment and operating cost perspectives. Furthermore, an SSF process significantly limits the loss of fermentable sugars caused by separation and transfer of sugars from the hydrolyser into fermenter as in an SHF process [107]. For ethanol production from JA tubers through SSF, the main technical challenge is the identification of the most efficient inulinases that are capable of facilitating hydrolysis, together with an appropriate yeast for fermentation.

The simultaneous hydrolysis and fermentation of JA tuber has been reported in a batch operation mode, using a co-culture of *Aspergillus niger 817* and *S. cerevisiae 1200* [136]. The ethanol concentration was 10.4% (v/v) for 15h fermentation period; the theoretical yield was 92%. Ge and Zhang (2005) attempted to use a newly isolated exoinulinase-hyperproducing strain, *A. niger* SL-09, coupled with *S. cerevisiae* Z-06 to ferment ground Jerusalem artichoke tubers into ethanol in a batch process. The ethanol concentration was as high as 19.5% (v/v) for 48h fermentation with a conversion efficiency of 90% [137]. The high ethanol concentration obtained in the finished fermentation broth is desired as it may significantly reduce the energy cost of the subsequent distillation step thereby improving economic feasibility [107].

The employment of an appropriate mixture of enzymes together with a fermentative micro-organism, achieves simultaneous hydrolysis and fermentation of Jerusalem artichoke tubers for improved ethanol production. However, these processes involving two species with diverse operating conditions pose challenges to process optimization. Both the hydrolytic enzyme or micro-organism and the fermentative micro-organism being operated under sub-optimal conditions may compromise the yield of ethanol produced. Another effort is to use yeasts that express inulinase activity to achieve simultaneous saccharification and fermentation [107]. Inulinase genes were actively expressed in fermentative yeasts such as *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* and applied in ethanol production

from JA tubers [138], [139]. With appropriate bioengineering and process optimizations, these organisms could be the prime candidates for commercial production of ethanol from inulin.

2.15.3 Production of inulinases

Inulinases are produced by different microorganisms, including bacteria, fungi and yeasts. [140]. The *Aspergillus* spp. are among the best-known producers of inulinases, while the strains of *A. niger* have been most investigated for inulinase production and characterization [140]. The production of both exo and endoinulinase by *Aspergillus ficuum* JNSP5-06 was investigated by Jing et al. (2003) [141]. Optimal fermentation conditions were found to be: inulin, 2%; yeast extract, 2%; $(NH_4)H_2PO_4$, 0.5%; NaCl, 0.5%; $MgSO_4.7H_2O$, 0.05%; $ZnSO_4.7H_2O$, 0.01%; initial pH 6.5 leading to inulinase activity of 0.55 IU/mL.

An *A. niger* strain, isolated from compost soil samples, produced both extra- and intracellular inulinases, which displayed identical pH and temperature optima with maximal activity observed at pH 4.3 and 4.4, temperature 55 and 56°C, respectively [142]. A strain of *A. niger* isolated from soil samples showed great capacity to produce extracellular inulinase. The optimum pH of the purified enzyme for inulin hydrolysis was found between 4.0 and 4.5 and the optimum temperature at 60 ^oC [143]. The inulinase to be applied in this study is a commercial endoinulinase isolated from *A. niger*.

Kluyveromyces spp. are also versatile sources of inulinases. A partially purified exoinulinase produced from K. *fragilis* was reported to be optimally active at pH 5.0 and 45 ^oC [144]. Inulinase produced and characterized from K. *marxianus* showed optimum pH and temperature at 5.0 and 50 ^oC respectively [145]. Even though there is a remarkable variability observed in the inulinaes from the various strains of K. *marxianus,* the inulinases only show moderate thermostability which limits their potential for commercial application [140].

The inulinase production levels in bacteria are not as impressive as those of the yeast and fungi. However, the ability of bacteria to thrive even at elevated temperatures has encouraged efforts to isolate bacterial strains capable of producing high amounts of thermally stable inulinase [140]. Inulinase produced from some stains of *Arthrobacter ureafaciens* was reported to be stable up to 70 ^oC except it was only activated by temperatures above 45 ^oC

[146]. Purified exoinulinase from *Streptococcus salivarius* demonstrated optimum pH and temperature at 6.0 and 50 ^{o}C repectivley [147].

Higher temperature optimum is an important factor for commercial application of these enzymes in IOS or fructose production from inulin. At these high temperatures (usually 60 ^{o}C and above), proper solubility of inulin is ensured, and microbial contamination of the inulin substrate is minimized [148]. Higher thermostability of the enzyme introduces some economic advantage as lower amount of enzyme is required to meet a production target due to increased activity at the elevated temperature [140]. Out of the many inulinases from fungi, yeast and bacteria, only few of them have optimum temperature of 60 ^{o}C or higher [149]–[153]. Although inulinases of bacterial origin have high thermostability, the low production levels places a limitation on their application at industrial levels [148]. Between the *Kluyveromyces* spp. and the *Aspergillus* spp. which are the most versatile yeast and fungal sources of inulinases, the *Aspergillus* spp. demonstrates the better thermostability [148].

2.16 Potential use of the biorefinery residues as livestock feed

The residues obtained after the inulin/IOS and protein co-production from Jerusalem artichoke are most likely to contain sugars, protein, ash, fatty acids [111], together with insoluble fibres that consists of cellulose, hemicellulose and lignin [154]. In a report, weaned pigs were fed diets containing Jerusalem artichoke tubers for 28 days. Significant weight gains were observed in the animal with improved feed efficiency [155]. It is therefore evident that some revenue could be generated from the application of the residues in animal feed apart from being used for bioenergy production.

2.17 Techno-economic survey of alternative production routes to short-chain fructose-containing oligosaccharides

While there is some substantial economic data in literature affirming the viability of commercial scFOS production from sucrose, the same cannot be said about IOS production from Jerusalem artichoke inulin. The economic viability of the application of Jerusalem artichoke in a biorefinery concept is still unclear, probably because the crop has not been widely cultivated and exploited on a commercial scale. However, there are some economic

estimations for the cultivation and use of Jerusalem artichoke for biorefinery application that highlight its economic potential.

2.17.1 Techno-economics of scFOS production from sucrose

In an economic evaluation by Vaňková et al. (2008), a process flowsheet was developed for large scale production of scFOS from sucrose using immobilized β-fructofuranosidase [95]. The cost estimations for scFOS production were done taking into consideration the desired production rate and type of scFOS (10 000 tonnes per annum of powdery FOS or 23 600 tonnes per annum of 43% scFOS syrup). The concentrated by-product made up of unconverted sucrose, glucose and fructose was sold at 0.50 €/kg. Prices of industrial and food sucrose substrates were 0.46 €/kg and 0.86 €/kg respectively. More than 97% of the raw material cost for enzyme immobilization was attributed to the cost of the resin for immobilization. However, immobilization equipment accounted for only about 6% of the total equipment cost for the production of immobilized enzyme.

In another study by Mussato et al. (2015), a comparative economic analysis was done on three methods of scFOS production from sucrose namely: Submerged fermentation using free cells (FCF), Submerged fermentation using immobilized cells (ICF) and solid-state fermentation. A production target of 200 tonnes per annum of scFOS was attained with the fermentation by-products (sweet protein) sold as animal feed at € 0.75/kg. The Total plant direct cost (TPDC) for the solid-state fermentation was the least (5.8 M€), due to the exclusion of the expensive fermenter and that of ICF was the highest due to the inclusion of extra equipment for immobilization (6.8 M€).

All three processes were found to be economically feasible, since their Net present values (NPV) were greater than zero and their Internal rate of return (IRR) values were also greater than the then prevailing interest rate of 7%. Even though the total capital investment and total operating cost for the ICF (13.1 M€) was greater than the FCF (12.3 M€), the ICF showed greater productivity and consequently a higher profit margin (1.99 M€ and 1.49 M€ for ICF and FCF respectively) due to the associated advantages of immobilization. In this case, the cost effectiveness of immobilization was able to offset the additional cost of immobilization.

The NPV and IRR values of ICF were higher than that of the FCF affirming the economic advantage of ICF over the FCF process.

An economic analysis was also done by Mathew Shedlock, a former student of Stellenbosch University [27]. In this study, economic estimations were carried out on various configurations of the two-stage submerged fermentation method of scFOS production from sucrose using free enzyme system. A production target of 2000 tonnes per annum of scFOS was determined as the minimum for an economically viable scFOS production facility in South Africa.

2.17.2 Economic potential of Biorefinery application of Jerusalem artichoke tubers

In a report by Johansson et al. (2015), for the biorefinery use of the crop to be economically viable, the carbohydrates in the tubers need to be used to produce high value platform chemicals [115]. A preliminary economic analysis carried out on Jerusalem artichoke as a biorefinery crop for producing proteins, rubisco protein, succinic acid and methane. It was estimated that the biomass production cost (cost of harvest, transport, seeds and mechanical row cleaning) was within the range of 3800 to 6000 €/ha. Results have shown that fermentation of Jerusalem artichoke tubers yields 3060 – 11000 l/ha of bioethanol, as the primary/sole application of its carbohydrate content, which is competitive compared to yields from sugarcane (6471 l/ha) and corn (4182 l/ha). However, prioritised production of ethanol from Jerusalem artichoke may not be financially attractive as it is not a high value commodity [156].

2.18 Conclusions

The following conclusions can be drawn from the literature review.

1. It is rather difficult to point out from literature a single immobilization method that is best suited for immobilization of β-fructofuranosidase for scFOS production on a large scale. Because each method has its own advantages and challenges. A well-informed choice can be made after empirical testing of a few potentially suitable methods, taking into consideration the unique properties of the engineered FFase that is the focus of the present study.

2. Two potentially efficient methods for immobilizing β-fructofuranosidase are:
   - » Entrapment using calcium alginate beads because of the simplicity of the immobilization process and cheapness of the immobilization material.
   - » Adsorption using anion exchange resins because of the simplicity and reversibility of the immobilization process, allowing for the anion exchange resin to be reused severally for the enzyme immobilization allowing for some savings on the cost of the immobilization material

   Essentially the two immobilization methods do not involve the use of coupling agents like glutaraldehyde which are said to be carcinogenic and not advisable for food grade applications. Experimental testing of these immobilization techniques is required to determine which is more appropriate for the enzyme of interest.

3. The technical and functional advantages of the immobilized β-fructofuranosidase enzyme over the free enzyme counterpart have been exclusively studied and proven. However, insufficient comparisons have been made regarding the economic viability of the scFOS production process, using the two enzyme systems. It is therefore inconclusive as to whether the immobilized enzyme system could be more economically viable than the free enzyme system under all circumstances or not, since this is also dependent on the characteristics of the particular enzyme of interest [141]. There is therefore the need for a techno-economic evaluation of the free and immobilized systems of scFOS production to ascertain if indeed the technical advantages of immobilization translate into economic advantage.

4. Biorefinery concept sometimes provides the platform for a more economical production of certain products by increasing revenue generation while minimizing the total cost of production by coextraction of other products alongside the main product. It is anticipated that commercial production of IOS from Jerusalem artichoke tubers may stand a greater chance of sustainability and economic feasibility when coupled with protein extraction in a biorefinery concept, considering that both substances are high value products.

5. The residual inulin and carbohydrates are a potential source of fermentable sugars for ethanol production and may enhance the economic feasibility and sustainability of the

biorefinery. Alternatively, these residues may also be applied in anaerobic digestion for biogas production or sold off as animal feed. The preferred option for valorisation of JA tuber residues after IOS extraction, is to be identified with techno-economic modelling.

6. There has not been any investigation on the economic feasibilities of scFOS production from sucrose compared to IOS production from JA tubers in a biorefinery. As competing products in the marketplace and alternatives to each other, it is expedient to conduct a comparative techno-economic analysis to determine which product offers the better profitability.

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Chapter 3

3 Research Aim and objectives

3.1 Aim

The aim of this study is to undertake process improvements and a comparative assessment of the economic feasibilities of sucrose and Jerusalem artichoke tuber as feedstocks for scFOS and IOS production respectively. This involves firstly, an attempt to minimize the production cost of scFOS by (i) exploring and optimizing the free and immobilized enzyme systems for sucrose conversion to scFOS, and (ii) optimization and investigation of integrated processes for the co-production of IOS and protein (nutraceuticals), bioenergy (ethanol or biogas) and/or animal feed (extraction residues) from Jerusalem artichoke tubers in a biorefinery concept.

3.2 Objectives

1. Immobilization of β-fructofuranosidase using three suitable support materials namely: Amberlite IRA 900 and Dowex marathon MSA, both anion exchange resins, and calcium alginate beads. The enzymes immobilized on the three supports will subsequently be characterised in terms of enzyme activity recovery and enzyme immobilization efficiency.

2. Comparison of the performance (product yields) of the immobilized enzymes to the free enzyme, for the production of scFOS with a chemical composition identical to Actilight®.

3. Assessing the re-usability of the immobilized enzyme and the regeneration capacity of the support materials used for the immobilization procedure.

4. Comparative techno-economics study of scFOS production from sucrose using the free and immobilized enzyme systems.

5. Optimization of the conversion of inulin in the inulin-rich substrates resulting from the alternative scenarios of inulin and protein co-extraction from JA tubers, into IOS a high value marketable product, through the application of endoinulinase enzyme.

6. Economic evaluation of various biorefinery scenarios applicable to the conversion of Jerusalem artichoke tubers, and comparison of the effective IOS production costs in such scenarios to the best economic cases for scFOS production from sucrose.

Chapter 4

4 Amberlite IRA 900 versus calcium alginate in immobilization of a novel, engineered β-fructofuranosidase for short-chain fructooligosaccharide synthesis from sucrose

Published research paper

Title: Amberlite IRA 900 versus calcium alginate in immobilization of a novel, engineered β-fructofuranosidase for short-chain fructooligosaccharide synthesis from sucrose

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Short summary

Objectives 1, 2 and 3 entailing the immobilization of the β-fructofuranosidase on suitable support materials, investigating and benchmarking the performance of the immobilized enzyme with the free enzyme counterpart is presented in this chapter (CHAPTER 4). This was done to achieve some amount of process improvement on the scFOS production process in the attempt to minimize the cost of scFOS production (CHAPTER 5) and allow a fair comparison with the cost of production of its counterpart IOS (CHAPTER 7). The β-fructofuranosidase enzyme was immobilized by adsorption onto the Amberlite IRA 900 and Dowex marathon MSA ion exchange resins and by entrapment in the calcium alginate beads. The success of the immobilization was determined by estimation of the immobilization parameters, for which the Dowex marathon MSA recorded the least favourable results and therefore, the rest of the investigation was continued with only the Amberlite IRA 900 ion exchange resin and the calcium alginate beads. The key finding was that, among the two immobilized enzymes, Amberlite IRA 900 demonstrated the better reusability (12 cycles of re-use) and consistency of product composition whereas the calcium alginate beads produced the higher scFOS yield of 59%. Immobilization therefore demonstrated the efficient use of the biocatalyst.

Declaration by the candidate:

With regard to Chapter 4, pg. 59 - 83, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Planning of experiments, execution of experiments, interpretation of results and compilation of chapter	80

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Declaration by co-authors: The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 4, pg. 59 - 83,
2. no other authors contributed to Chapter 4, pg. 59 - 83, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 4, pg. 59 - 83, of this dissertation.

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Amberlite IRA 900 versus calcium alginate in immobilization of a novel, engineered β-fructofuranosidase for short-chain fructooligosaccharide synthesis from sucrose

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Abstract

The immobilization of β-fructofuranosidase for short-chain fructooligosaccharide (scFOS) synthesis holds the potential for a more efficient use of the biocatalyst. However, the choice of carrier and immobilization technique are key to achieving that efficiency. In this study, Calcium alginate (CA), Amberlite IRA 900 (AI900) and Dowex Marathon MSA (DMM) were tested as supports for immobilizing a novel engineered β-fructofuranosidase from *Aspergillus japonicus* for short-chain fructooligosaccharide (scFOS) synthesis. Several immobilization parameters were estimated to ascertain the effectiveness of the carriers in immobilizing the enzyme. The performance of the immobilized biocatalysts were compared in terms of the yield of scFOS produced and reusability. The selection of carriers and reagents was motivated by the need to ensure safety of application in the production of food-grade products. The CA and AI900 both recorded impressive immobilization yields of 82% and 62% respectively, while the DMM recorded 47%. Enzyme immobilizations on CA, AI900 and DMM showed activity recoveries of 23%, 27% and 17% respectively. The CA, AI900 immobilized and the free enzymes recorded their highest scFOS yields of 59%, 53% and 61%, respectively. The AI900 immobilized enzyme produced a consistent scFOS yield and composition for 12 batch cycles but for the CA immobilized enzyme, only 6 batch cycles gave a consistent scFOS yield. In its first record of application in scFOS production, the AI900 anion exchange resin exhibited potential as an adequate carrier for industrial application with possible savings on cost of immobilization and reduced technical difficulty.

4.1 Introduction

The increasing awareness and consumption of healthy and functional foods has promoted research into the applications and production dynamics of short-chain fructooligosaccharides (scFOS). Short-chain fructooligosaccharides consisting of 1-kestose (GF2), nystose (GF3) and 1^F-fructosylnystose (GF4) are nutraceuticals with application in the food and drug industries. They offer functional benefits such as minimal caloric value, non-cariogenicity and prebiotic properties [1], [2]. Recently, scFOS was found to alleviate postprandial glycaemic response in adults when blended with maltitol in the formulation of sugar free foods [3]. Other applications of scFOS include: infusion in Indian gooseberry via osmotic treatment to increase the antioxidant activity of the fruit [4] and addition to frozen dough to increase the proof volume and subsequently the moisture retention capacity and texture of bread [5].

The industrial production of scFOS relies more on the polymerisation of sucrose via the fructosyltransferase reaction of β-fructofuranosidases (FFase), rather than the hydrolysis of inulin by inulinases [2]. These industrial β-fructofuranosidases are usually of fungal origin, belonging to *Aspergillus*, *Aureobasidium* and *Pencillium* genera [1]. Applying the free FFase in batch scFOS production is often the process that assures the highest scFOS levels (55-60% [$w/w_{total\ sugars}$]) [6], but the main drawback is the difficulty in the recovery and reuse of the biocatalyst. Another challenge lies in the need to rapidly terminate the reaction by deactivation of residual enzyme once the desired product yield and composition are attained [7]. Immobilization offers a solution by allowing the recovery and reuse of expensive enzymes, and simultaneously may enhance their pH and temperature stability [8]–[11]. The heterogeneous nature of an immobilized catalyst allows for its application in various reactor types and provides the option of continuous operation [12], [13].

Several immobilization techniques and support materials have been developed in the quest to efficiently utilize the biocatalyst. These range from covalent immobilization on SHIRASU porous glass [14], chitosan particles [8], [15], methacrylamide-based polymeric beads [16] and ceramic membranes [6] to entrapment in calcium alginate (CA) beads [1], [17] and adsorption onto anion-exchange resins [18], [19]. A few of these have recorded impressive results on the laboratory scale, while others cannot be readily applied in food processing due

to reservations about the use of potentially poisonous coupling agents like glutaraldehyde [8], [20]. There is no universal immobilization technique or enzyme carrier that is efficient for all enzymes. The most effective immobilization methods used to achieve good bond strength result in some degree of activity loss by changing the protein conformation, while the methods that maintain high enzyme activity are not able to strongly bind the enzyme to the carrier materials. Therefore, a compromise between activity and bond strength is usually reached [21].

Amberlite IRA 900 (AI900) and Dowex Marathon MSA (DMM) are highly porous strong base anion exchange resins that have recorded satisfactory results in immobilizing FFase [18]. They are both composed of a styrene–divinylbenzene matrix [18] and have the advantage of being applicable in food processing, due to their non-toxic nature [7]. However, the AI900 possesses trimethyl ammonium functional groups while the DMM has quaternary amine functional groups.

FFase related enzymes have isoelectric points (pI) between pH of 4 to 5 (predominantly negatively charged), hence strongly attracted to anion exchange resins [22], [23]. CA gel has also been reported to be efficient in immobilizing FFase due to its biochemical inertness, mechanical stability, strength and the availability of interstitial pores and spaces for trapping the enzyme [9], [11], [20].

The enzyme used in this study was an engineered, recombinant *Aspergillus japonicus* β-fructofuranosidase (EC 3.2.1.26 belonging to glycoside hydrolase family 32 of the carbohydrate-active enzymes database) that displayed improved specific activity, thermostability and relief from product (glucose) inhibition [24]. Previous work using the soluble form of this enzyme determined the optimal conditions for scFOS production [24]. The same conditions were applied to the study of the immobilized enzymes as many reports have shown the free β-fructofuranosidases and their immobilized counterparts to have optimal activities under identical conditions [15], [16], [25]–[28]. Neither the influence of immobilization on scFOS production nor the recycling of this novel enzyme have been evaluated hitherto.

This study tested adsorption onto ion exchange resins (AI900 and DMM) as well as entrapment in CA beads in order to evaluate potential alternatives for application of this new FFase in industrial scFOS production. To the best of the authors' knowledge, there is no data on scFOS production using the AI900 immobilized enzyme. Neither has a comparison been made between adsorption and entrapment immobilization of a particular β-fructofuranosidase for scFOS production with considerations to the scFOS composition. The CA and AI900 immobilized enzymes demonstrate great potential as adequate replacements for the free enzyme in industrial production of scFOS.

4.2 Materials and methods

4.2.1 Materials

HPLC-grade standards (D-glucose, D-fructose and D-sucrose), HPLC-grade sodium acetate, Pierce BCA protein quantification kit, sodium alginate powder, DMM and AI900 resins were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). The fructooligosaccharide standards (1-kestose, nystose and 1^F-fructofuranosylnystose) were purchased from Wako Chemicals GmbH (Neuss, Germany). HPLC-grade 50% NaOH solution was obtained from Fluka (Merck, Darmstadt, Germany). All other chemicals and reagents used were of analytical grade purity as purchased.

4.2.2 Analyses

4.2.2.1 Protein quantification

The protein concentrations of samples were determined by the bicinchoninic acid (BCA) assay adapted to 96 well micro-titer plates. A standard deviation of less than 10% was allowed due to the nature of the protein estimation process [29].

4.2.2.2 HPLC: high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Sugar analyses for glucose, fructose, sucrose, 1-kestose, nystose and 1^F-fructosylnystose was performed by the method described by Sorensen and Brodbeck [30]. Sugar quantification was performed by comparing peak areas to those of external reference standards.

4.2.3 Enzyme production

A *Komagataella* (*Pichia*) *pastoris* strain expressing the four amino acid substitution variant of the *Aspergillus japonicus fopA* FFase (GenBank accession number AB046383) under the *GAP* promoter [24], was kindly donated by the department of Microbiology, Stellenbosch University, South Africa. The production procedure was carried out as described by Anane et al. (2016) [31]. In this study, basal salt medium (BSM) supplemented with *Pichia* trace elements (PTM_1) was used in a 1.3L BioFlo 110 bioreactor (Eppendorf- New Brunswick, Hamburg, Germany). A dissolved oxygen (DO)-stat feeding strategy (30% set-point) with 50% (w/v) glycerol as substrate at a rate of 4.57 g/h for 72 h was initiated after the initial glycerol batch phase. The pH was maintained at 5.0 with 25% NH_4OH and aeration at 1 volume air per initial volume fermentation broth per minute (vvm). The fermentation broth was centrifuged at 8000 x *g* for 20 min to obtain the crude supernatant containing the extracellularly secreted β-fructofuranosidase. No further purification steps were applied to the crude supernatant.

4.2.4 Preparation of CA beads

Preparation of 3% (w/v) alginate beads was carried out as described by Anis et al. [11], with minor modifications. CA beads were prepared at 25 ^oC by mixing 0.75 g sodium alginate in 16.7 mL reverse osmosis purified water to form a slurry. A 8.3mL Crude enzyme solution (1870 U) was then added while ensuring thorough, continuous mixing. The alginate-enzyme mixture was extruded dropwise through a 25-gauge needle into 250 mL 0.1 M $CaCl_2$ solution using a peristaltic pump. The $CaCl_2$ solution was continually stirred using a magnetic stirrer to produce a slight vortex. The droplets solidified forming spherical beads in the solution. Further stirring was allowed for 30 min, after which the beads were collected using a filter funnel and washed with 50 mM sodium acetate buffer, pH 5.0. The beads were cured by placing them in 50 mM sodium acetate buffer pH 5.0 at 4 ^oC for 24 h.

4.2.5 Adsorption of enzyme on anion exchange resins

Portions of the DMM and AI900 resins were pre-treated by washing first with water for 30 min, followed by 0.2 M HCl for 30 min. Resins were then washed with four bed volumes of 4% (w/v) NaOH for 2 h followed by washing with water until a supernatant pH of 9.0 was obtained. The resins were spread to dry at room temperature. Five hundred milligrams each of pre-treated (OH^-) and untreated resin (Cl^-) were incubated separately in 50 mL enzyme

solution (5200 U of enzyme in 0.05 M sodium acetate buffer pH 5.0) at 62 °C, 150 rpm. The supernatants were sampled hourly for 12 h. The procedure was repeated at 25 °C for both resins.

4.2.6 Activity assay of free and immobilized enzymes

The free enzyme activity assay was performed as described previously [32]. For the estimation of the immobilized enzyme activity, masses of immobilized enzyme (1 g CA beads and 230 mg anion exchange resins) were incubated in 10 mL 10% (w/v) sucrose solution (prepared by dissolving 10 g sucrose in 100 mL of 0.05 M sodium acetate buffer pH 5.0) at 40 °C and 120 rpm for 1 h. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol of glucose per minute when reacting enzyme with 10% (w/v) sucrose at 40 °C for 1 h.

4.2.7 Enzyme adsorption profile on ion exchange resins

A 500 mg mass of the untreated resin (Cl^-) was incubated in 50 mL enzyme supernatant at 25 °C, pH 5.0 and 150 rpm. The supernatant was sampled at 4 h intervals for 12 h and after 24 h. The procedure was repeated at 62 °C. The entire process was performed on both DMM and AI900 resins.

4.2.8 Immobilization parameters

The enzyme immobilization parameters were determined as follows:

$$Immobilization\ yield\ (Y_i) = \frac{(A_i - A_f)}{A_i}\ x\ 100 \qquad \{a\}\ [9]$$

Where A_i (total initial enzyme activity) is the total number of activity units detected in the starting enzyme solution used for the immobilization process. A_f is the number of activity units measured in the filtrates and washing solutions after immobilization.

$$Immobilization\ efficiency\ (E_f) = \frac{A_m}{(A_i - A_f)}\ x\ 100 \qquad \{b\}\ [21]$$

Where A_m is the number of activity units detected on the support material after immobilization and washing.

$$Activity\ recovery\ (\mathrm{A}_r) = \frac{A_m}{A_i}\ x\ 100 \qquad \{c\}\ [21]$$

The activity assays for the estimation of these parameters were all carried out under the same conditions.

4.2.9 scFOS production

Masses of immobilized enzymes (5 g CA beads and 400 mg AI900) were reacted with 50 mL 60% (w/w) sucrose solution, pH 5.0 at 62 ^{o}C for 12 h at 120 rpm. The reaction mixture was sampled at 3 h intervals to estimate the amount and composition of scFOS produced.

4.2.10 Reusability of immobilized enzyme

The same conditions were used as for a single batch of scFOS production, however after 6 h of incubation the beads were transferred to fresh sucrose solution. The process was repeated for 15 batch cycles.

4.2.11 Ion exchange resin regeneration studies

Five hundred milligrams of untreated resin (Cl^-) was incubated in 50 mL enzyme supernatant (115 mg/L protein) at 62 ^{o}C and 25 ^{o}C separately at pH 5.0 and 150 rpm. The supernatant was sampled after 12 h and protein concentration estimated. The resins were regenerated by washing with 4 bed volumes 4% NaOH solution and dried at room temperature. The process was repeated for 10 regeneration cycles.

4.2.12 Statistical analysis

All data were measured in triplicates from three independent runs. Standard deviations have been provided where necessary. Statistical analysis of data was carried out by one-way and two-way analysis of variance (ANOVA) using Statistica for Windows software version 13.2 and statistical significance was considered when p-value < 0.05.

4.3 Results and discussion

An engineered β-fructofuranosidase, secreted into the culture supernatant by a recombinant *K. pastoris* strain, was immobilized by adsorption and entrapment methods and evaluated in batch reactions for the production of scFOS from sucrose. These immobilization carriers were selected based on their food grade safety status and the simplicity of associated immobilization procedure.

4.3.1 Immobilization data: enzyme entrapment in CA beads

Table 4.1 gives a summary of the immobilization parameters for the enzyme immobilization. The CA beads recorded immobilization yield (Y_i) and immobilization efficiency (E_f) of 82 ± 3.28% and 28 ± 1.40% respectively. The immobilization yield gives an indication of how much enzyme in the starting solution was retained in the beads during the immobilization process, based on the difference in enzyme activity in the solution before immobilisation, and the $CaCl_2$ and washing solutions after immobilization. This value is comparable to published data [9]. Activity is mainly lost via protein leakage during the calcium alginate gel formation, as droplets of the sodium alginate-enzyme make contact with the $CaCl_2$ solution [20]. Accompanied by the protein loss was leakage of water from the beads during the cross-linking. This was observed in the 12% difference in mass of the initial alginate mixture and the total mass of the CA beads formed. Dashevsky (1998) [33] reported a similar occurrence where 44% water was lost from 1% (w/v) alginate beads, indicative of the inverse relation between amount of water loss and alginate density. A comparison of the immobilization yield and the percentage protein immobilized (Table 4.1) highlighted that the immobilization yield based on activity far exceeded the percentage of protein immobilized. This difference is linked to the deactivating effect that $CaCl_2$ has on the β-fructofuranosidase [34], leading to a deceptive impression of a high immobilization yield. In this case the percentage protein immobilized was a better reflection of the immobilization yield.

The immobilization efficiency is an estimation of the percentage of the immobilized enzyme that is detected on the support material. This exposes any inherent activity loss to the CA immobilized enzyme as a result of changes in the microenvironment of the enzyme and deactivation of any active sites. It also accounts for apparent loss in activity because of diffusional limitations on the substrates and products in and out of the CA beads respectively. The activity recovery (A_r) is of more practical value – it exposes the combined effects of protein leakage during bead formation, diffusional limitation and the inherent loss of enzyme activity. A_r is therefore a more adequate measure of the effectiveness of a particular immobilization process. As a result, the activity recovery value as obtained for the CA beads (23 ± 1.84%) was significantly lower than the corresponding immobilization yield. A report by Fernandez-Arrojo et al. [20] showed similar results, where their activity recovery was 4-fold

lower than the immobilization yield. The diffusional limitation in the beads increases with bead swelling, density and diameter [35].

Table 4.1: Summary of immobilization parameters for calcium alginate beads and ion exchange resins

	AI900		DMM		CA
Immobilization temp, °C	**25**	**62**	**25**	**62**	**25**
Immobilization yield, %	42 ± 2.52	62 ± 2.48	21 ± 0.84	47 ± 1.88	82 ± 3.28
Immobilization efficiency, %	33 ± 2.31	43 ± 2.15	53 ± 2.65	36 ± 1.80	28 ± 1.40
Activity recovery, %	14 ± 1.12	27 ± 2.16	13 ± 1.04	17 ± 1.36	23 ± 1.84
Protein loading[C]	4.8 ± 0.43	8.1 ± 0.73	3.2 ± 0.29	5.7 ± 0.51	4.6 ± 0.41
Fraction of total proteins immobilized, %	41± 2.05	74 ± 3.70	32 ± 1.60	60 ± 2.70	37 ± 1.67

[C] mg of protein per g of dry ion exchange resin; mg of protein per g of dry sodium alginate powder, AI900 – Amberlite IRA 900, DMM – Dowex Marathon MSA, CA- Calcium alginate

4.3.2 Immobilization data: enzyme adsorption onto anion exchange resins:

The β-fructofuranosidase in the crude culture supernatant was also immobilized by direct adsorption onto the two anion exchange resins of choice (AI900 and DMM). For each resin, the adsorption was carried out at two temperatures (25 °C and 62 °C). The two temperatures were selected to minimize any form of protein inactivation or interactions due to many temperature variations as the enzyme preparation is not completely purified [36]. A summary of the immobilization results is displayed in Table 4.1. The highest immobilization yield of 62 ± 2.48% was associated with the AI900 resin at 62 °C. Vaňková et al. (2008) [7] reported a similar immobilization yield of 65% for a fructosyltransferase enzyme on DMM at 12 °C. The immobilization efficiencies for the ion exchange resins (33 ± 2.31, 36 ± 1.80, 43 ± 2.15 and 53 ± 2.65%) were significantly low, mainly due to the presence of mass transfer restrictions owing to the porous nature of the ion exchange resins despite adsorption being a surface phenomenon. There is usually little or no conformational change of the enzyme structure

during immobilization by adsorption [37]. However, the efficiency values were greater than that of the CA beads. This was most likely due to the reduced diffusional restrictions on substrates and products to and from the enzyme active sites, respectively, by reason of the resins' smaller particle sizes. The immobilization efficiencies obtained in this study were higher than the 11.1 and 13.9% (Table 4.2) reported in literature for similar types of anion exchange resins.

Table 4.2: The present study and published works on some carriers applied in immobilizing β-fructofuranosidase for scFOS synthesis

Carrier	(Y_i), %	(A_r), %	(E_f), %	scFOS[a]	Reference
Adsorption					
Amberlite IRA 900	42	14	33	53.0	(This study)
Dowex Marathon MSA	65	*	11.1	*	[18]
Amberlite IRA 900	*	*	13.9	*	[18]
Diaion HPA 25	*	*	*	55	[27]
Entrapment					
Calcium alginate	82	23	28	61.4	(This study)
Calcium alginate	*	*	*	55	[28]
Dried alginate-entrapped enzymes (DALGEEs)	50	*	*	46	[20]
Calcium alginate	*	*	*	67.75	[11]
Gluten	*	*	*	61	[38]
Covalent binding					
Eupergit C	*	*	96	57	[25]
Chitosan-coated magnetic nanoparticles	*	*	80	59.5	[15]
methacrylamide-based polymeric beads	100	*	*	60	[16]
Chitosan beads	*	*	*	48	[8]
Chitosan beads	88	*	54	55	[26]
chitosan beads	*	*	*	42.79	[11]
Chitosan beads	82	*	42	59	[36]
Simultaneous cross linking and covalent binding					
Chitosan beads	81	*	93	72.2	[9]
Simultaneous cross linking and entrapment					
Calcium alginate	90	*	52	68.5	[9]

[a] % (g of scFOS/g of sucrose); * Value not reported in literature

4.3.2.1 Effect of resin pre-treatment and temperature on resin adsorption capacity

The effect of resin pre-treatment with HCl and NaOH on the extent of protein adsorption was statistically insignificant ($p > 0.05$), while temperature had a significant effect ($p < 0.05$) (Table S1 of Appendix A). Further adsorption studies proceeded on the untreated resins as it is the economically advisable option to avoid the cost of chemicals for pre-treatment. Figure 4.1 shows the adsorption profile of the anion exchange resins at the selected temperatures. AI900 adsorbed the highest amount of protein (74%) at 62 ^{o}C followed by the DMM (60%) at the same temperature after 24 h. Comparatively low amounts of protein (41 and 32% for AI900 and DMM, respectively) were adsorbed at 25 ^{o}C. In all four scenarios, the highest adsorption rate was recorded within the first 3 h, followed by a gradual decline until saturation point was reached after 24 h. This behaviour is typical of many adsorption processes [39]. Even though the anion exchange resins (AI900 and DMM) demonstrate high adsorption capacities at high temperatures, prolonged exposure of the free enzyme to elevated temperatures is not advisable due to protein denaturation and subsequent loss of enzyme activity [40].

Considering that the mean particle size, moisture holding and total exchange capacities of the AI900 and DMM are almost identical, the higher adsorption capacity and activity recovery obtained with the AI900 could probably be due to the difference in the nature of the interactions between the charged functional groups of the resin and the enzyme [41]. The AI900 and DMM possess trimethyl ammonium and quaternary amine functional groups respectively. The values of enzyme loading obtained in this work were within the ranges reported in literature [42]. Due to its superior performance, further experimental work involving ion exchange resins was performed with AI900.

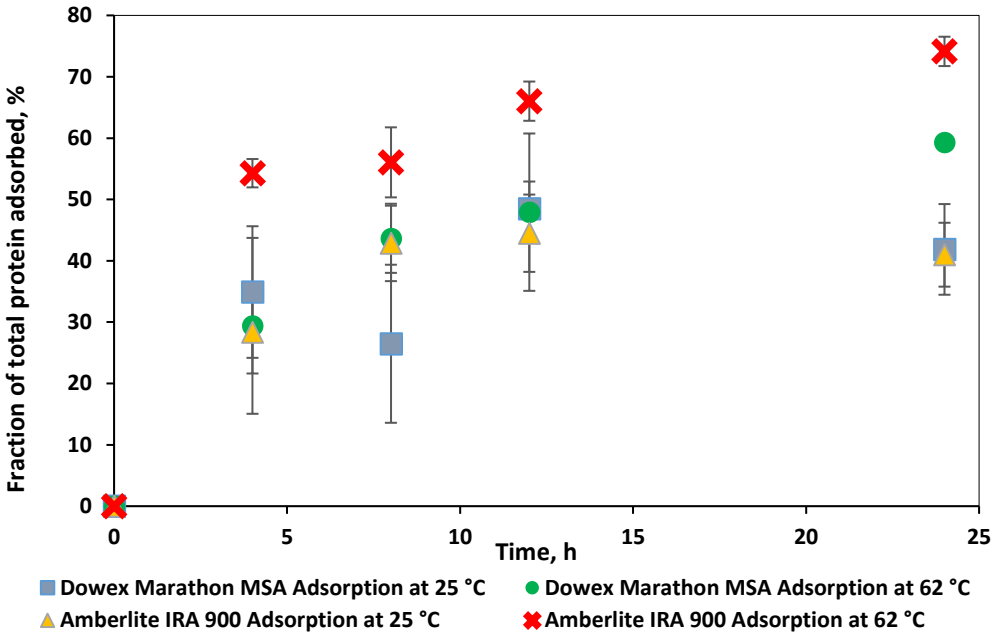

Figure 4.1: Effect of temperature on the percentage protein adsorbed by untreated Dowex Marathon MSA and Amberlite IRA 900

4.3.2.2 Regeneration capacity of AI900

The regeneration capacity of AI900 was investigated at 25 °C and 62 °C by repeated cycles of enzyme immobilization and resin regeneration. AI900 maintained high adsorption capacities (averages of 35% and 64% of total proteins adsorbed at 25 °C and 62 °C, respectively) for 10 cycles of reuse (Figure 4.2). There was no statistical difference in the extents of protein adsorption for the various cycles ($p < 0.05$), at each adsorption temperature. However, there was a trend towards increased adsorption capacity at 62 °C for every subsequent regeneration cycle. This could be due to the lower selectivity of OH^- ions (resulting from NaOH regeneration), which facilitates ion exchange with the enzyme molecules compared to Cl^- ions (originally on the resin), which have higher selectivity [43]. These data support the regeneration and reuse of the AI900 resin beyond the effective life span of the immobilized enzyme. The weak nature of the ionic bonds formed between the enzyme and the ion exchange support offers the luxury of regeneration, which may provide potential for alleviating the cost of immobilization. The ion exchange resin became darker in colour with each cycle of adsorption (data not shown). In a report by Yun and Song (1996) [27], a similar occurrence was noticed for prolonged operation of their ion exchange column. This could be

linked to the cumulative effect of the irreversible adsorption of dissolved impurities in the crude enzyme solution [44]. The good regenerative capacity of the ion exchange resins also indicates good mechanical strength, a desirable property for catalyst carrier if it is to be used on an industrial scale.

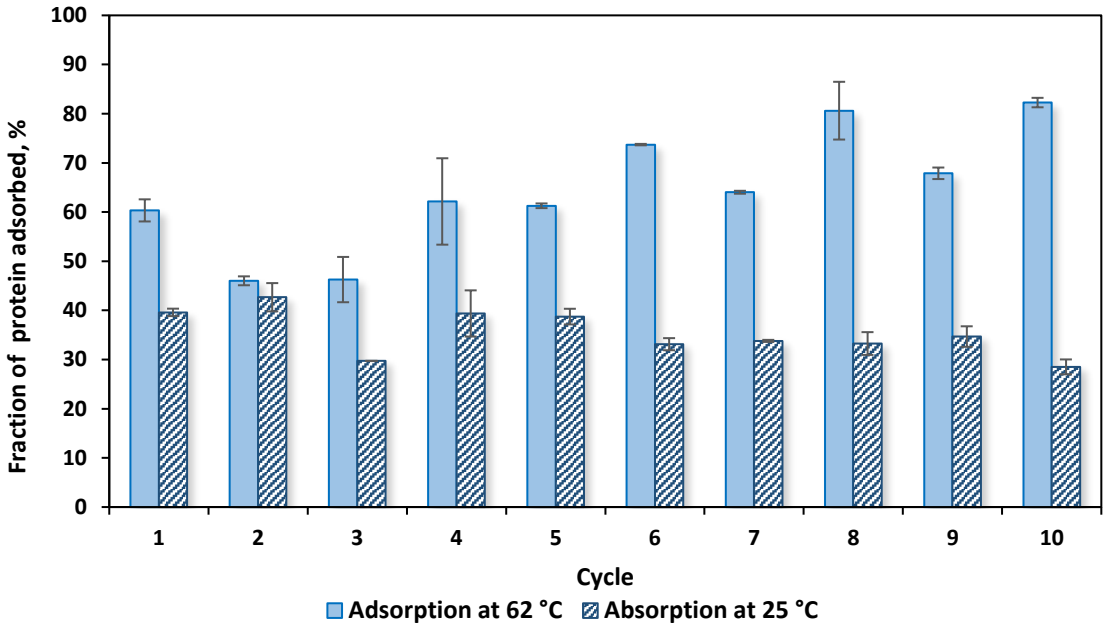

Figure 4.2: Effect of temperature on regeneration capacity of Amberlite IRA 900

4.3.3 scFOS synthesis from sucrose

The performances of immobilized (CA beads and AI900) and free enzyme preparations were evaluated in terms of their ability to produce scFOS with compositions in the ranges of 35-40% GF2, 50-55% GF3 and 5-10% GF4 (w/w_{scFOS}). These compositions were selected as similar scFOS preparations have clinical data to support health claims [45], [46]. Displayed on Table 4.3 are the percentage profiles of the saccharides compositions for the three enzymes during a 12h scFOS synthesis reaction. In all three enzymes, the scFOS compositions most closely resembling the target prebiotic were obtained after 6 h, at which time scFOS production peaked (61.4, and 58.8% {$w/w_{total\ sugars}$}) for the free enzyme and CA immobilized enzyme respectively). The AI900 on the other hand showed the peak scFOS production of 53% ($w/w_{total\ sugars}$) after 9 h of reaction.

Three hours into the reaction, the free and CA immobilized enzymes recorded their highest GF2 percentages of 42.0% and 32.7% ($w/w_{total\ sugars}$) respectively, while the AI900 immobilized enzyme recorded only 18.0% GF2 and later peaked at 21.6% after 6 h. At the same time (3 h), 2.1, 6.3 and 15.3% ($w/w_{total\ sugars}$) GF4 were detected for the free, CA and AI900 immobilized enzymes, respectively. These data indicate an accelerated conversion of GF2 to GF3 and GF3 to GF4 in the immobilized enzymes especially the AI900 immobilized enzyme relative to the free enzyme. The formation of GF4 seems to be favoured by the immobilization probably due to the diffusional limitation, allowing for a longer enzyme-GF3 contact time for conversion to GF4 [27]. A similar observation was reported in a semi-batch production of scFOS using cells of *A. pullulans* immobilized in CA beads [47]. The GF3 concentrations followed a similar trend, except the latter reached peak percentages of 31.1, 24.4 and 20.3% ($w/w_{total\ sugars}$) for the free, CA and AI900 immobilized enzymes, respectively after 9 h.

In all three enzymes, the conversion rate of sucrose was rapid at the early stages (0-6 h) of the reaction. This declined significantly later in the reaction, partly due to the product inhibition effect of accumulated glucose, a well-known phenomenon associated with FFases [9], [15]. The conversion rate of sucrose was the highest with the free enzyme with 7.4% ($w/w_{total\ sugars}$) of unconverted sucrose remaining after 12 h. This indicates an expectedly higher rate of reaction in the case of the free enzyme, due to the absence of mass transfer limitations [38]. In addition to the mass transfer limitation in the immobilized enzymes, resulting in the slower sucrose conversion rate, the increased enzyme-GF3 contact time may have also increased the substrate competition of GF3 with sucrose, by reducing the number of active sites available for sucrose conversion at a given time. The AI900 immobilized enzyme showed the slowest conversion rate of sucrose with 9.8% ($w/w_{total\ sugars}$) of unconverted sucrose remaining after 12h. The reaction mechanism of this enzyme finds consistency with that proposed by Jung et al. [48] for *Aspergillus japonicus* and related enzymes. A sufficient amount of the preceding oligosaccharide was required as substrate for the formation of its homologue with an additional fructose moiety. The scFOS percentages obtained for the immobilized enzyme in this study are within range of those reported in literature (Table 4.2).

Table 4.3: Percentage composition (w/w) of total sugars and scFOS produced by the free β-fructofuranosidase, calcium alginate and Amberlite IRA 900 immobilized enzymes during a 12 h reaction at 60 °C. 60% (w/w) sucrose pH 5.0 as substrate and 10U/g of sucrose enzyme dosage.

Time, h	Percentage of total sugars							Percentage of scFOS		
	F	G	GF	GF2	GF3	GF4	scFOS	GF2	GF3	GF4
Free β-fructofuranosidase										
0	*	*	100.0	*	*	*	*	*	*	*
3	1.1	25.0	13.2	42.0	17.4	1.3	60.6	69.2	28.7	2.1
6	1.7	28.7	8.2	28.3	27.9	5.2	61.4	46.0	45.5	8.5
9	2.0	29.3	8.1	20.9	31.1	8.7	60.6	34.4	51.2	14.3
12	1.1	31.7	7.4	19.6	27.6	12.6	59.7	32.8	46.2	21.0
Calcium alginate immobilized enzyme										
0	*	*	100.0	*	*	*	*	*	*	*
3	1.3	25.2	21.2	32.7	16.3	3.3	52.3	62.5	31.1	6.3
6	2.3	29.1	9.9	24.1	23.9	10.8	58.8	41.0	40.6	18.4
9	2.9	32.7	8.3	16.3	24.4	15.4	56.1	29.1	43.5	27.4
12	3.4	34.0	7.7	13.4	23.4	18.1	54.9	24.5	42.6	32.9
Amberlite IRA 900 immobilized enzyme										
0	*	*	100.0	*	*	*	*	*	*	*
3	1.2	18.0	45.6	18.0	11.8	5.4	35.2	51.1	33.6	15.3
6	1.8	27.4	19.4	21.6	18.5	11.3	51.4	42.0	36.0	22.0
9	2.6	32.7	11.7	19.9	20.3	12.8	53.0	37.6	38.2	24.2
12	3.0	37.6	9.8	14.5	17.1	18.1	49.6	29.1	34.4	36.5

F-fructose, **G**-glucose, **GF**-sucrose, **GF2**-1-kestose, **GF3**-nystose, **GF4**-1^F-fructosylnystose, **scFOS**- short-chain fructooligosaccharides, ***-not detected

4.3.4 Reusability of immobilized β-fructofuranosidase

The extent to which enzymes immobilized by entrapment or adsorption were reusable, was investigated by applying the same batch of immobilized enzyme in a series of sequential reactions. A high degree of reusability is desired to offset the additional immobilization costs for the feasible industrial production of scFOS using the immobilized enzyme system. The

performances of β-fructofuranosidase immobilized with CA or AI900 carriers were compared for 15 consecutive cycles. For the CA immobilized enzyme, an average scFOS yield of 61.3% ($w/w_{total\ sugars}$) was obtained for the first six cycles, although the scFOS composition was inconsistent. Proportions of the higher degree of polymerisation scFOS species, GF3 and GF4, decreased with each successive cycle until the 6th cycle, beyond which only GF2 was detected (Figure 4.3A). These data are evidence of reduced enzyme activity, most likely as a result of protein loss from the alginate matrix or thermal enzyme deactivation [49]. Prolonged exposure to high temperature and agitation usually causes the collapse of the bead structure, thereby resulting in the rapid rate of protein loss from the alginate beads [11]. It has been reported that whole cells of *A. flavus,* containing fructosyltransferase immobilized in CA beads, maintained high scFOS production yields (an average of 65.37%) for seven successive cycles, without a significant loss in activity [11]. Alginate beads may be more efficient for immobilization of mycelia rather than enzymes due to the larger molecular size of the former [1].

In the case of the AI900 immobilized enzyme (Figure 4.3B), an average scFOS yield of 48% ($w/w_{total\ sugars}$) was obtained for 12 cycles, with a consistent scFOS composition (p-value > 0.05). After this, a substantial decline in yield was observed in the 13th cycle. The first two cycles recorded high scFOS yields (52% $w/w_{total\ sugars}$) after which the yield reduced to an almost consistent value for the other 10 cycles. This could be due to the loss of some activity stemming from desorption of loosely bound enzymes during agitation. The AI900 therefore exhibited better reusability (consistency of yield and production composition) compared to the CA beads. Product consistency is desired in order to maintain a consistent quality of product in terms of functional/prebiotic properties and sweetness thereby alleviating any additional steps to achieve consistency of product composition. [50].

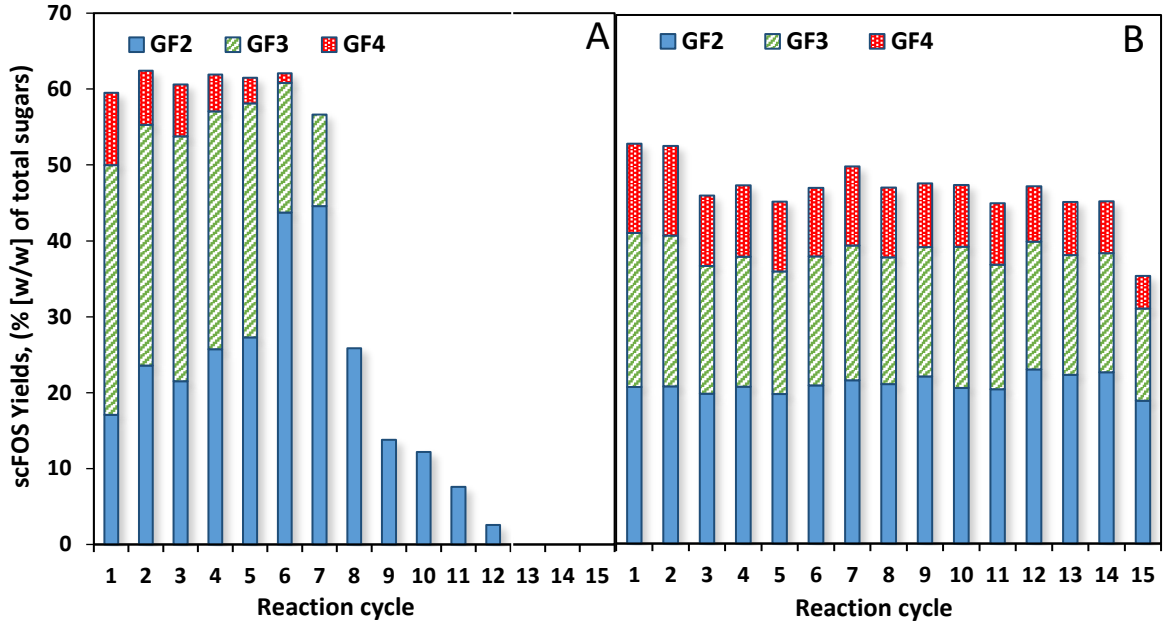

Figure 4.3: Production of scFOS by reutilization of immobilized enzymes. ***A****-Calcium alginate immobilized enzyme.* ***B****-Amberlite IRA 900 immobilized enzyme. 60% (w/w) sucrose pH 5.0 as substrate, 62 °C, 120 rpm and 6 hours per reaction cycle. GF2-1-kestose, GF3-nystose and GF4-1^F-fructosylnystose*

4.4 Conclusion

CA and AI900 were evaluated as supports for a novel β-fructofuranosidase based on the efficiency of immobilization, performance of the immobilized enzymes against the free enzyme in scFOS production and reusability of the immobilized enzymes. Results revealed the AI900 and CA as adequate supports for immobilizing the novel enzyme for scFOS production. Where the consistency of the product scFOS is not a priority, the CA immobilized enzyme offers impressive yield (61%) and satisfactory reusability. However, considering the tendency of enzyme leakage from the beads with every recycle, the AI900 could be the more suitable alternative. The AI900 immobilized enzyme has the advantage of support regeneration and production of a consistent scFOS yield and composition per recycle which is desirable in order to assure a consistent scFOS taste and prebiotic effect in an industrial production process. Unlike other carriers, the simplicity of immobilization technique associated with the CA and AI900 could reduce the technical difficulties, which may be associated with industrial application of the immobilized β-fructofuranosidase. The low material costs, efficient enzyme activity recoveries, reusability of enzyme and carrier and comparable scFOS production levels

make the immobilized enzymes investigated in this study good technical alternatives to free enzyme catalyzed reactions.

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Chapter 5

5 Comparison of immobilized and free enzyme systems in industrial production of short-chain fructooligosaccharides from sucrose using techno-economic approach

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Short summary

The objective 4, which entailed the techno-economic study on the free and immobilized enzyme systems, was investigated in this chapter (CHAPTER 5). Three scFOS production systems namely: the Amberlite IRA and calcium alginate immobilized enzyme and the free enzyme systems were simulated in the Aspen plus® software using the experimental data in CHAPTER 4. Further economic analysis was conducted on the enzyme systems based on these simulations, to ascertain economic viability. All three systems were found to be economically viable, although the free enzyme system was marginally more profitable. The key finding here was that the savings on the cost of enzyme as a result of immobilization was offset by the costs incurred as a result of immobilization, thus effectively given no net benefit to immobilisation. The economic feasibilities of the scFOS production scenarios explored here were ultimately compared with those obtained for IOS production from JA tubers (CHAPTER 7) to ascertain which product has superior profitability.

Declaration by the candidate:

With regard to Chapter 5, pg. 84 - 120, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Simulation work, interpretation of results and compilation of chapter	80

The following co-authors have contributed to Chapter 5, pg. 84 - 120:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
J.F. Görgens	jgorgens@sun.ac.za	General discussions and revision of chapter	10
Mohsen Mandegari	mandegari@sun.ac.za	Review of simulation, revision of chapter	10

Signature of candidate:……

Date:……..

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 5, pg. 84 - 120,
2. no other authors contributed to Chapter 5, pg. 84 - 120, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 5, pg. 84 - 120, of this dissertation.

Signature	Institutional affiliation	Date
	Stellenbosch University	
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Comparison of immobilized and free enzyme systems in industrial production of short-chain fructooligosaccharides from sucrose using techno-economic approach

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Abstract

Short-chain fructooligosaccharides (scFOS) are nutraceuticals with numerous applications in the food and pharmaceutical industries. The production of scFOS using the immobilized biocatalyst offers some functional and technical advantages over the free enzyme counterpart. To investigate the economic potential of the immobilized enzyme system relative to the free enzyme system, a techno-economic comparison was conducted on three methods of scFOS production (powder and syrup forms) at capacity of 2000 tonnes per annum (tpa) by enzymatic synthesis from sucrose, i.e. the free enzyme (FE), calcium alginate immobilized enzyme (CAIE) and Amberlite IRA 900 immobilized enzyme (AIE) systems. These processes were simulated in Aspen Plus to obtain the mass and energy balances and estimate the operating and capital costs, followed by economic evaluation and sensitivity analysis. Profitability analysis showed all three systems are economically viable as their associated minimum selling prices (MSP) were well below the scFOS market price of 5$/kg. However, the FE system was the most profitable with the least MSP of 2.61 $/kg, because the savings on cost as a result of enzyme immobilization could not offset the additional costs associated with immobilization. Sensitivity analysis demonstrated total operating cost, fixed capital investment and Internal Rate of Return (% IRR) have the greatest effects on the MSP. Furthermore, syrup form of scFOS production leads to 29% less MSP, compared to powder form. In addition, the plant capacities of 5000 and 10000 tpa were also studied and 10% and 16% reductions on MSP were obtained.

5.1 Introduction

Biorefining, which is the integral conversion of several bio-based materials into an array of valuable products and energy, has in recent times been immensely researched as a way of alleviating the many global concerns associated with high dependence on fossil reserves [1]. In order to make biorefineries economically viable and sustainable, much research has been directed towards development of more efficient conversion technologies and biorefinery pathway configurations [2]. Utilizing carbohydrates and sugars primarily containing glucose, fructose, xylose and sucrose for production of biofuels and biochemicals through a biorefinery is *so-called* as first generation (1G) biorefinery. However, there have been concerns about competition with food production industries for the use of the biomass and arable lands [3]. Second generation (2G) biorefinery is more challenging biorefinery approach which is conversion of biomass and waste material to variety of bio-based products [4].

Sucrose, which is mostly produced from sugarcane[5], [6] is considered one of the readily available sugars and a precursor to a wide variety of bulk and fine chemical products, most notable of which include: 5-(hydroxymethyl)furfural (HMF), furfural, lactic acid, gluconic acid and short-chain fructooligosaccharides (scFOS) [7], [8]. The markets for these bio-based products are gradually getting established with some already dominating global production.[9] Furfural and 5-(hydroxymethyl)furfural (HMF), which are intermediates to polymer precursors, antifungals and pharmaceuticals, are conveniently produced by the acid catalyzed conversion of sucrose under specified conditions [10]. Bio-based furfural constitutes up to 100% of the total market volume of 300,000 – 700,000 tonnes per annum (tpa) with a selling price of 1-1.45 $/kg. The selling price of bio-based HMF which constitute 20% of total HMF volume of 100 tpa was estimated to be around 2.65 $/kg [9].

Lactic acid is another important platform chemical obtained by the anaerobic fermentation of sucrose [11]. It finds various applications in the food and pharmaceutical industries and also polymerized into poly-lactic acid (PLA). The selling price is estimated at 1.45 $/kg. The bio-based lactic acid constitutes 100% of the total market volume, which stands at about 472,000 tpa due to the absence of an identical fossil-based product [9], [12]. Gluconic acid, which is a complexing and acidifying agent used by pharmaceutical and food industries, can

also be obtained by the oxidation of glucose from sucrose [10]. The global production of gluconic acid was estimated at 100,000 tpa and it is produced almost solely via biotechnological processes [10].

Short-chain fructooligosaccharides (scFOS) have numerous applications in the food and pharmaceutical industries due to their functional properties such as low calorific content, non-cariogenicity and tendency of reducing the triglycerides, cholesterol and phospholipids levels in humans [13]. They also possess bifidus-stimulating properties and facilitate the adsorption of vitamins and minerals in the colon [14], [15]. Short-chain fructooligosaccharides have received much research attention due to the increasing global demand in correspondence to the increasing awareness towards weight control, immune system activation, aging rate, gut and heart health [16]. This high value prebiotic resembles sucrose in terms of taste (sweetness) and physicochemical properties, and therefore could be an adequate replacement for sucrose, providing supplementary functional benefits to the consumer, while yielding higher revenue to the manufacturer [15].

As a component of functional foods, scFOS has a Generally Regarded As Safe (GRAS) status from the Food and Drug Administration (FDA), USA [16]. The market price of scFOS was estimated in 2008 to be around 4 €/kg [17], recently reported prices are as high as 150 €/kg, depending on the desired quality and quantity [18]. In 1995 the global demand for scFOS was around 20 000 tpa [18]. Presently, the global demand for prebiotics (mainly scFOS, inulin, isomaltooligosaccharides, polydextrose, lactulose and resistant starch) is about 167,000 tonnes equivalent to 390 million Euros [18]. This reflects a well-established global market for functional sweeteners with considerable growth expected annually. Japan has been a dominant consumer in the prebiotics market with annual demands of 69,000 tonnes [19]. Australia, China, India, Indonesia, Korea, Malaysia and Singapore have also been identified to have the most attractive markets and growth potential for the prebiotic market due to their increasing prebiotic and healthy food consumption consciousness [20]. Short-chain fructooligosaccharides have also gained prominence in the US and Europe with daily intakes of up to 4g and 11g per person, respectively [21]. The US holds 50% of the global scFOS and inulin market share [16]. The European sale of prebiotics was estimated to be $1.17 billion

with Netherlands, France, Germany and UK contributing two-thirds [16]. scFOS have been marketed around the world under different trade names, some of which include: Actilight®, Profeed®, Oligo-Sugar®, NutraFlora®, Beneshune™ P-type and Meioligo® [18].

Short-chain fructooligosaccharides occur naturally in some plants like asparagus, onion and Jerusalem artichoke [22]. However, the commercial production of scFOS relies on either the transfructosylation of sucrose or inulin hydrolysis. Fructooligosaccharides from sucrose account for more than 50% of the total scFOS market due to the availability of sucrose sources [18]. Commercial production of scFOS from sucrose using a fructosyltransferase or β-fructofuranosidase enzyme can be classified in two main categories namely: the production of scFOS using free enzyme, and the production of scFOS using the immobilized enzymes or immobilised cells producing these [23], [24].

Using a conventional, free enzyme system, scFOS have been produced commercially in a batch process by mixing the biocatalyst with 50-60% (w/v) sucrose solution at pH 5.5-6.0, and temperature of 50-60 ^{o}C with continuous mixing for 4-20 hours [24]. To stop the enzymatic activity, the reaction mixture (mostly made up of scFOS, fructose, glucose and unconverted sucrose) is heated to 90 ^{o}C for a period of 30 minutes followed by cooling below 50 ^{o}C and a series of purification steps [24]. The major drawback of this system is the high cost of using a fresh biocatalyst for each batch reaction, and the need to rapidly terminate the enzyme activity when the desired product yield and composition is reached [25].

Alternatively, the immobilized cell or enzyme system can be deployed [25]–[27]. This system provides better temperature and pH stability for the enzyme and allows for recovery and reuse of the biocatalyst. However, scFOS yields are usually lower than the free enzyme counterpart, due to the loss of activity from mass transfer limitation and inherent loss of activity during the enzyme immobilization process [28]–[30]. Another possible short coming is that unlike most conventional heterogenous catalysts, the immobilized biocatalyst in many cases experiences a substantial loss in activity after a limited number of reuses typically between 7 to 15 cycles of reuse [28], [31], [32]. As a result of the technical and functional advantages of the immobilized over the free enzyme reported in literature, many authors

have proposed that the industrial application of an immobilized β-fructofuranosidase in scFOS production may offer economic advantages over the free enzyme [16], [33]–[35].

Simulation development and techno-economic evaluation of the scFOS production have not been reported wieldy. Vaňková et al. [25] conducted simulation and costing of a 10 000 tonnes per annum scFOS production facility using immobilized enzyme in a two-stage submerged fermentation process. Another research also compared the economics of scFOS production via single stage submerged fermentation vs solid state fermentation [36]. To the authors' knowledge, a detailed economic comparison of scFOS production using the free and immobilized enzymes via the two-stage submerged fermentation is yet to be carried out.

In this study, the production of scFOS from sucrose by transfructosylating reaction of β-fructofuranosidase enzyme was considered [32]. Three different enzyme processes (free, Calcium alginate and Amberlite IRA 900 immobilized enzymes) are compared for scFOS production to investigate the economic prospects of the immobilized enzyme over the free enzyme counterpart. To achieve this, mass and energy balances are constructed for all three systems based on simulation models developed in Aspen Plus. Furthermore, cash flow analyses are conducted and economic indicators determined. Different scFOS production capacities are also simulated to evaluate the effect of production scale on profitability. In addition, economic sensitivity analyses are also carried out to investigate the effect of involve parameters on the economic viability.

5.2 Materials and methods

Production capacity plays a significant role in the economic viability of the industrial processes. However, care must also be taken not create abundance in the market while maintaining economic viability. A production target of 2000 tonnes per annum (tpa) of scFOS was set as the base production scale considering the rising demand for scFOS in South Africa and the world at large. This capacity also proposed by Shedlock [37] as the minimum scale for an economically viable scFOS production facility. However, production capacities of 5000 and 10000 tpa were also simulated and compared with the base case in terms of investment and profitability. The selected process for this study was the two-stage submerged fermentation which is the most commonly applied system in commercial scFOS production [18]. Based on

the selected process descriptions, process simulations were built in Aspen Plus® V8.8 software (Aspen Technology Inc., USA), using experimental data from a previous study [32] and data from the other published works [25], [36], [37].

5.2.1 Simulation development

Among the different process simulation softwares, Aspen Plus has been applied broadly for simulation of a large number of biorefineries [2]. The Aspen Plus property database compiled by the National Renewable Energy Laboratory (NREL, USA) provides properties of some components that are not found in the Aspen Plus database [38]. Table S1 of Appendix B shows the list of the user defined components specified in the scFOS production processes simulated in this work. With the help of the Aspen Plus property method tool, NRTL method was determined as the appropriate property method to predict the thermo-physical properties.

5.2.2 Economic Evaluation approach

The economic evaluations of alternative process scenarios were carried out in Microsoft Excel, by importing the mass and energy balance data from the Aspen Plus models. The cost evaluations were conducted in 2016 US dollar indices representative of process plants located in the USA. The cost of some equipment such as pumps, heat exchangers, compressors and flash drums were estimated by the Aspen Plus Economic Evaluator package. Other equipment such as the bioreactor, spray dryer, moving bed chromatography (SMB) column, evaporator and centrifuge were estimated by updating the cost and capacities of identical equipment from reported data [25], [36], [37] using the equipment-specific scaling exponents and indices [38]–[40].

The total purchased equipment cost forms the seed from which the fixed capital investment (FCI) and the total capital investment (TCI) were estimated. The FCI is a summation of the total direct cost (TDC) and the total indirect cost (TIC), whereas the TCI is a summation of the FCI, the working capital (WC) and the cost of land [38]. The WC was estimated as 5% of the FCI, whilst the cost of land was estimated as 8% of the total purchased equipment cost [40]. The total operating cost consisted of the variable operating cost (including raw material cost, utility cost and waste disposal cost) and the fixed operating cost (including labour and supervision cost, labour burdens and maintenance). Majority of the raw material costs were

obtained through vender quotes and reported literature [25], [36], [37]. Utility costs were extracted from Aspen Plus by specifying the type of utility applied to meet the energy demand of the various unit operations. The labour burden was estimated as 90% of the total labour and supervision costs, which were estimated from reported literature [39]. Maintenance was estimated as 3% of the inside battery limit (ISBL), while the property and insurance tax was estimated as 0.7% of the FCI [2]. The operating labour cost was calculated from salaries reported in literature [39].

A discounted cash flow rate of return (DCFROR) analysis was performed in Microsoft Excel for the various process scenarios based on the estimated capital and operating expenditures. The economic assumptions deployed in this study are outlined in Table 5.1. Since there is some uncertainty with regards to the selling price of scFOS, a more appropriate method of comparing the investigated economic scenarios is on the basis of the minimum scFOS selling price at an acceptable minimum internal rate of return (IRR) of 9.710% for the real term. The minimum selling price (MSP) is calculated by iterating the scFOS selling price until a net present value (NPV) of zero is obtained [41]. An economic sensitivity analysis was also performed to ascertain the effect of changing some key economic parameters on the minimum scFOS selling price.

Table 5.1: Economic assumptions

Description	Value
Plant financing equity	100% [42]
Plant life	25 years [41]
Annual operating hours	7920 hours
Acceptable minimum %IRR (real term)	9.7% [41]
Income tax	28% [41]
Annual depreciation rate	4%[41]
Plant salvage value	Zero [41]
Start-up time	Zero years [43]
Costing year	2016
Exchange rates	US$1 = 14.51 ZAR, €1 = 15.46 ZAR
Selling price of by-product sugars	0.2 $/kg [25]
Selling price of scFOS	5 $/kg [17]
Liquids treatment/disposal	5.9 $/ton [39]
Solids treatment/disposal	33.5 $/ton [39]

5.2.3 Process model description

In order to study the economic viability of the scFOS production via free and immobilized enzyme systems, three scFOS production scenarios were developed as follows: Scenario 1: scFOS production using the free enzyme (FE) system. Scenario 2: scFOS production using the calcium alginate immobilized enzyme (CAIE) system. Scenario 3: scFOS production using the Amberlite IRA 900 immobilized enzyme (AIE) system.

Scenario 1 can be considered as the base case scenario in the economic evaluation of the scFOS production process. Scenarios 2 and 3 were included to ascertain if indeed the

technical and functional advantages of β-fructofuranosidase immobilization for scFOS production translate into economic advantage.

The free enzyme system for scFOS production is mainly considered in two stages, namely: β-fructofuranosidase production and scFOS production. The former stage entails all the processes involved in the preparation of the enzyme inoculum, enzyme fermentation and partial enzyme purification (ultrafiltration). The scFOS production stage involves the application of the produced enzyme in the scFOS production reaction as well as all the scFOS purifications steps. Figure 5.1 provides a detailed process flow diagram of scFOS production with the free enzyme system. The immobilized enzyme systems (scenarios 2 and 3) are considered in three stages, namely: β-fructofuranosidase production, β-fructofuranosidase immobilization and scFOS production. The β-fructofuranosidase production and scFOS production stages have similar process flow configuration for all three scenarios, except for variations in flow rates and equipment sizes with each scenario due to the variation in enzyme and sucrose demands. In the case of the calcium alginate and the Amberlite IRA 900 immobilized enzyme systems, the β-fructofuranosidase immobilization stage entails all the processes involved in the preparation and activation of the enzyme carrier and the immobilization prior to application in the scFOS production reaction.

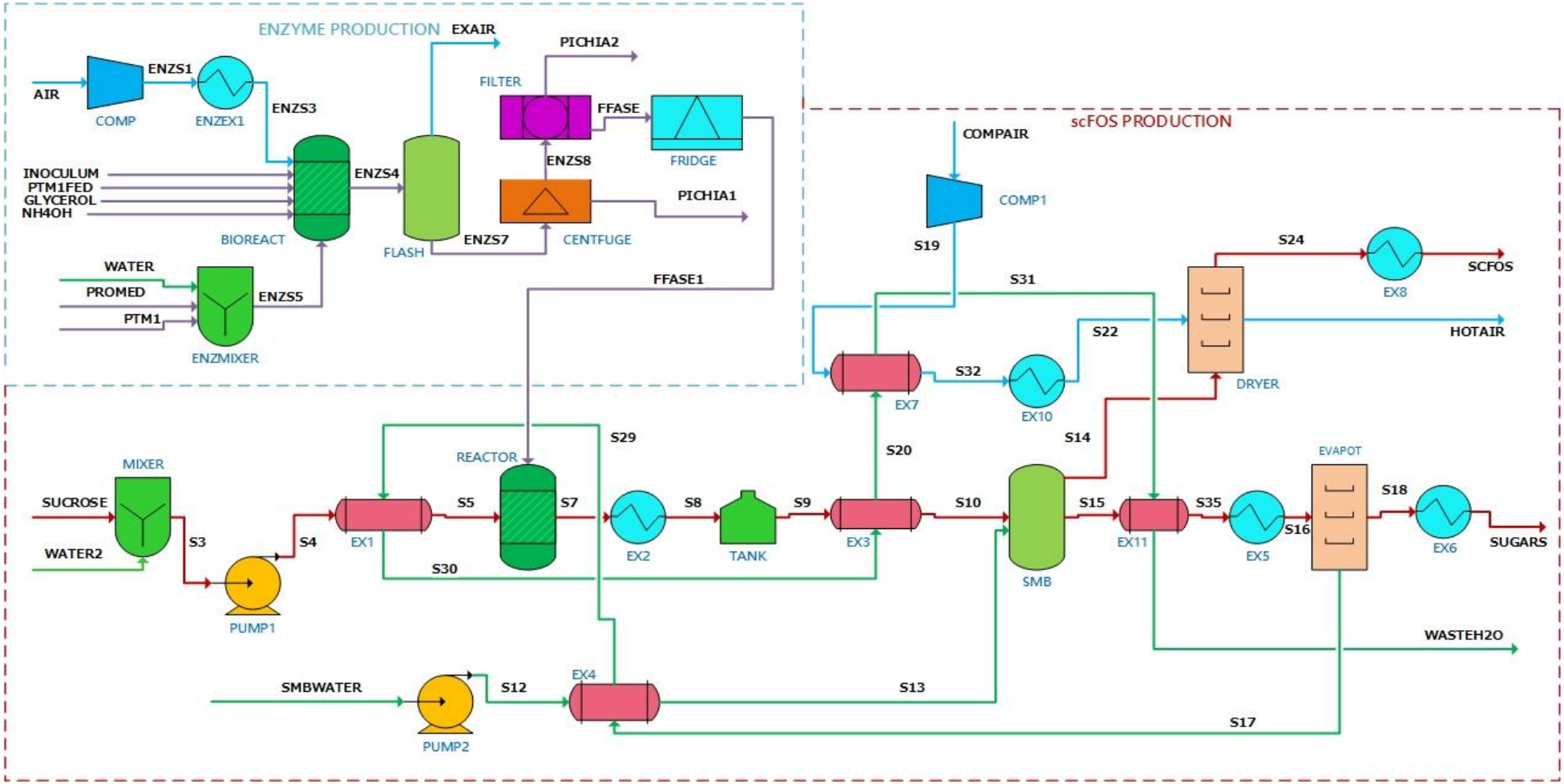

Figure 5.1: Process flow diagram for 2000 tpa scFOS production with the free enzyme system.

5.2.4 Free β-fructofuranosidase enzyme (FE) system

5.2.4.1 β -fructofuranosidase production stage

The enzyme production stage starts with the cultivation of pre-inoculum in the laboratory, which was simulated as described by Anane et al. (2016) [44]. The grown culture in the shake flask is carried through a series of scale-up of seed fermentations, until there is enough volume to serve as the seed inoculum for the 1000 L fermenter (BIOREACT in Figure 5.1). The seed inoculum is usually 5-10% of the initial fermentation volume [45]. Prior to inoculation, the bioreactor is filled with basal salt medium (stream ENZS5 in Figure 5.1) and sterilized by heating with high pressure steam to 141 ^{o}C for about 10 seconds followed by cooling to 30 ^{o}C and the addition of 1.6 L of *Pichia* trace salts (PTM1) solution (composition in Table S2 of Appendix B). The fermenter is then inoculated with the seed inoculum and maintained at 30 ^{o}C. It is essential to maintain the temperature at 30 ^{o}C, since it is the optimal for growth of the culture and the protein expression [44]. Large fluctuations in temperature could be detrimental to the growth of the organism. Simultaneously the culture is continuously agitated at 100-1000 rpm and supplied with compressed air from a compressor (COMP in Figure 5.1) at 0.4 bar and 1 vvm (volume of gas per volume of medium per minute). The agitation facilitates the dissolution of oxygen in the culture, while the aeration ensures mixing and oxygen transfer to the micro-organism in the culture. The dissolved oxygen (DO) is kept above 30% air saturation by cascading the level of agitation. The air supply rate is dependent on the rate of consumption of glycerol by the organism during growth.

The pH of the culture is maintained at 5 using a 25% ammonium hydroxide solution (stream NH_4OH in Figure 5.1), to allow for optimal growth and also to provide a nitrogen source for the organism. The batch culture is allowed to grow for ~24 hours, within which all the glycerol is consumed. This is evident when the DO concentration reaches 100% [44]. The biomass concentration is expected to be within the range of 90-150 grams of wet cells per litre of working volume at the end of the batch phase [44]. This is a prerequisite for the initiation of the next phase, which is the glycerol fed batch phase.

The glycerol fed batch phase is initiated by feeding 50% w/v glycerol feed (stream GLYCEROL in Figure 5.1) supplemented with 26.3 g/h of PTM1 trace salts (stream PTM1FED in Figure 5.1) at an initial rate of 2.34 kg/h. A DO-stat feeding regime is implemented where the DO is controlled at 30% with a cascade system and the feeding starts at a DO above 35% and stops below this value. The glycerol fed batch stage is allowed to proceed for 4-5 days. The fermenter is modelled in Aspen Plus using an RYIELD reactor where the desired wet cell and protein yields are specified. A schedule is required to maintain an efficient and optimised operation of the fermenter. The schedule shows times and durations for seed preparations and cleaning of equipment. The size of the main fermenter usually determines the sequence of seed inoculums to be prepared since the inoculum for the main fermenter has to be 5-10% of the initial fermenter volume. Figure S1 in Appendix B shows a schedule for the operation of the fermenter for a constant supply of enzyme to the scFOS production facility.

During the course of the fermentation, the enzyme is secreted extracellularly, together with other proteins, into the supernatant. Two main effluents exit the fermenter: The gaseous effluent (stream EXAIR in Figure 5.1) mainly composed of carbon dioxide, and nitrogen and the unused oxygen from the supplied air. The other stream is the fermentation broth containing the yeast cells, the secreted enzyme, proteins and metabolites (stream ENZS7 in Figure 5.1). The flash separator (FLASH in Figure 5.1) is included in the Aspen Plus model to achieve the phase separation of the gaseous components from the fermentation broth. However, its equipment cost is not included in the total equipment cost, since it is not required in reality.

The fermentation broth is sent to the centrifuge (CENTFUGE in Figure 5.1), where the separation of the yeast (stream PICHIA1 in Figure 5.1) cells from the supernatant containing the enzyme (stream ENZS8 in Figure 5.1) is achieved at an efficiency of 98% [25]. The supernatant is passed through an ultrafiltration unit (FILTER in Figure 5.1) where the residual yeast cells are removed. The purified enzyme supernatant (stream FFASE in Figure 5.1) is refrigerated at 4^{o}C and supplied to the scFOS production unit when required.

5.2.4.2 scFOS production stage

Results from previous works [32] obtained scFOS yield of 61% (w/$w_{total\ sugars}$) using the free enzyme and therefore was adopted for the industrial simulation as well. The 60% (w/v) sucrose solution is prepared by mixing sucrose at a feed rate of 442kg/h with 295 kg/h of water in a blending tank (MIXER in Figure 5.1). After thorough mixing, the sucrose solution is fed to the bioreactor (REACTOR in Figure 5.1). The type of reactor suitable for this application is the semi-batch continuously stirred tank reactor (CSTR). This is modelled in Aspen using the RYIELD reactor, by specifying the desired scFOS yield and composition. The partially purified enzyme supernatant (stream FFASE1 in Figure 5.1) from the enzyme production facility is supplied to the bioreactor containing the substrate solution. The reaction is allowed to proceed under constant agitation at a temperature of 62^{o}C for 6 hours. The heat exchanger EX1 in Figure 5.1 is included in the flowsheet to capture the energy required to heat up the reaction mixture and maintain it at 62^{o}C for the designated reaction period.

In the case of the free enzyme system, the reaction is terminated by quickly heating the reaction mixture to 90^{o}C for a brief period. The reactor product stream made up of scFOS, glucose, fructose and unreacted sucrose is sent the blending tank (TANK in Figure 5.1) to be cooled to room temperature [24]. Heat exchanger EX2 in Figure 5.1 is inserted to capture the cooling requirement of the mixture in the blending tank.

The scFOS is extracted from the mixture using a simulated moving bed chromatography column (SMB in Figure 5.1) with scFOS recovery efficiency of 95%. The SMB consists of four columns charged with a cation exchange resin Amberlite^{TM} 1320 Ca connected in series. The ion exchange resin is well adapted for separation of polysaccharides from di- and monosaccharides [25]. Water (stream S13 in Figure 5.1) is used as the eluent, and enters the SMB at 385 kg/h; columns are operated at 60^{o}C. The extract (mainly scFOS) represented by stream S14 in Figure 5.1 and the raffinate (mainly sucrose, glucose and fructose), represented by stream S15 in Figure 5.1; both exit the SMB at 550 kg/h and 575 kg/h respectively. scFOS stream is sent to the spray drier, the syrup is dried to obtain scFOS in powdered form (Stream S24 in Figure 5.1), with a purity of 95%. The spray dryer is represented in Aspen using a configuration of unit operation blocks (COMP1, EX7, EX10 and DRYER in Figure 5.1). The spray

dryer uses 7217 kg/h of hot air at 180 °C to dry the product which is sprayed into a long hollow column along with the hot air. The COMP1 compresses the air to the required pressure and flowrate; heat exchangers EX7 and EX10 raise the temperature of the air to the required temperature and the DRYER is used to achieve the phase separation. The raffinate made up of sucrose, glucose and fructose is sent to the evaporator, where it is dried at 100 °C to obtain a di- and monosaccharides content of 95% (stream S18 in Figure 5.1). The evaporator is also represented by the heat exchanger EX5 in Figure1 to obtain the heat requirement and a flash separator (EVAPOT in Figure 5.1) to achieve the desired separation. Some level of energy integration was effected in the process flowsheet to reduce the total energy consumption of the plant. The energy demand of the processing plant was optimized via pinch analysis using the Aspen Plus Energy Analyzer®.

5.2.5 Calcium alginate immobilized enzyme (CAIE) system

5.2.5.1 β-fructofuranosidase immobilization stage

The design of the immobilization facility for large scale production of alginate beads with entrapped enzyme was based on previous reports [15], [46]. Figure 5.2 shows a process flow diagram of scFOS production via the calcium alginate immobilized enzyme system. The alginate enzyme slurry is prepared in a 500 L mixing tank (MIXER2 in Figure 5.2) by mixing 11.5 kg of sodium alginate powder with 255 L of water and 128 L of the enzyme supernatant. The slurry (stream S37 in Figure 5.2) is fed to the bead making equipment (BEADMAK in Figure 5.2). The bead making equipment is made up of two chambers: the upper chamber is a 500L pressure vessel with a porous bottom fitted with numerous tiny orifices. The lower chamber is a 3000 L vessel containing 2400 L of 0.1M $CaCl_2$ solution.

The top of the upper vessel and the bottom of the lower vessel are both connected to a compressor (COMP2 in Figure 5.2) for supply of compressed air. Once the upper vessel is filled with the enzyme alginate slurry, it is closed tightly and pressurised with atmospheric air. The pressure forces the alginate slurry to be extruded through the orifices into the $CaCl_2$ solution as droplets. These droplets then undergo crosslinking to form the alginate beads. The bottom of the $CaCl_2$ vessel is sparged with air to enable a gentle stirring of the $CaCl_2$ solution to prevent the beads from coalescing during the hardening process. The entire process is carried

out at 25°C. The beads are fortified in 0.05M sodium acetate buffer solution pH 5.0 at 4°C for 12 hours, prior to charging into the scFOS reactor to increase their mechanical strength [28], [47]. The beads once loaded into the scFOS production reactor vessel are maintained for 6 batches of reaction cycles before discarded. Two hundred and twenty immobilization cycles (36 hours per cycle) are required to meet the scFOS production target of 2000 tpa.

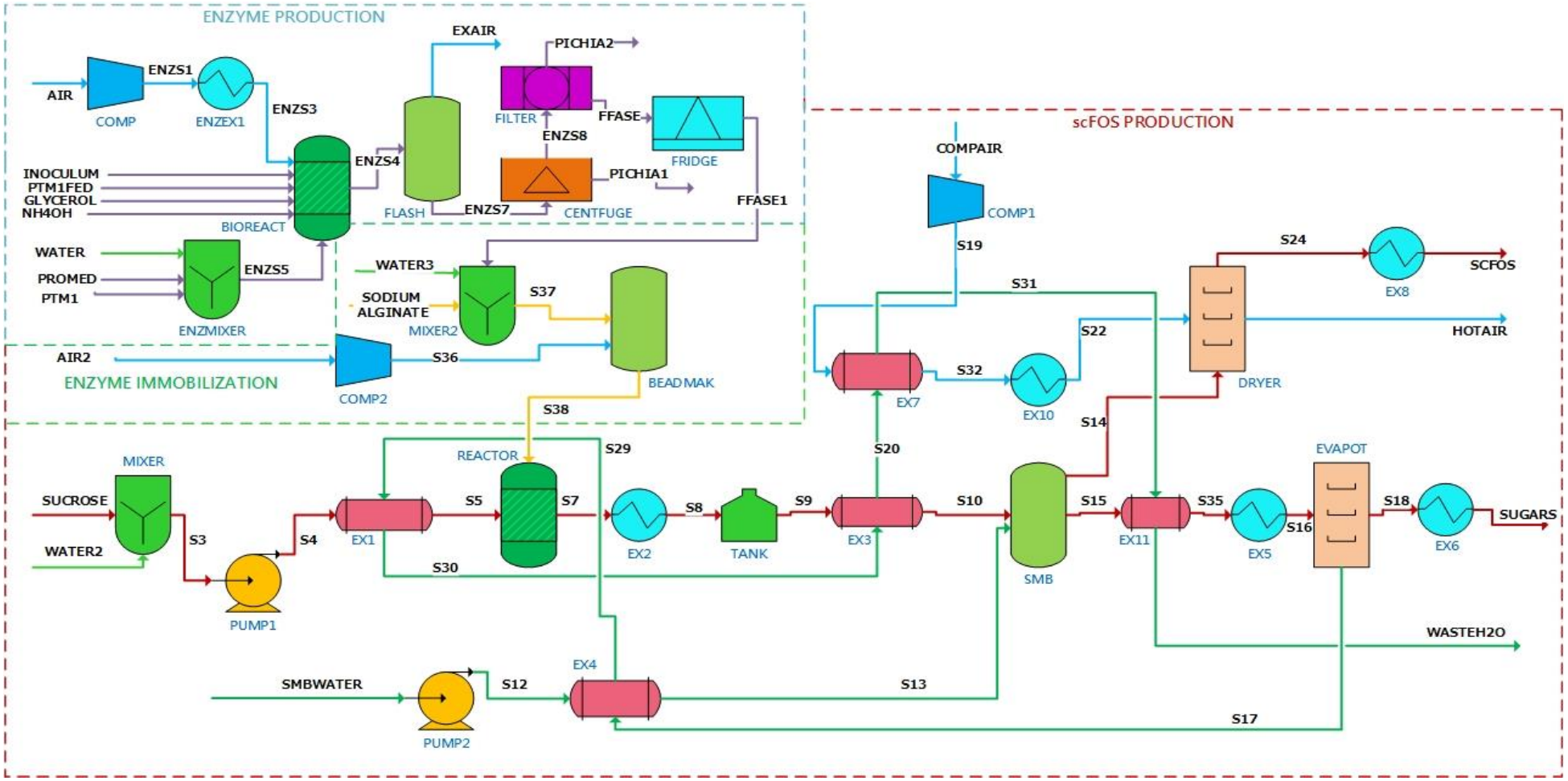

Figure 5.2: Process flow diagram for 2000 tpa scFOS production with the calcium alginate immobilized enzyme system.

5.2.6 Amberlite IRA 900 immobilized enzyme (AIE) system

5.2.6.1 β-fructofuranosidase immobilization stage

The immobilization process is carried out as described by Vaňková et al. (2008) [25]. Figure 5.3 shows a process flow diagram of scFOS production via the amberlite IRA 900 immobilized enzyme system. Six hundred and sixty litres of filtered supernatant is mixed with the ion exchange in a 1000L stirred stank (STIRTANK in Figure 5.3) at 25^{o}C for 12 hours. The heterogeneous mixture (stream S36 in Figure 5.3) is then passed through a filter (FILTER2 in Figure 5.3) to separate the Amberlite IRA 900 with immobilized enzyme from the residual enzyme supernatant, to be re-used during a subsequent immobilization process. The ion exchange resins with the immobilized enzyme are then washed to remove any unbound protein prior to application in the scFOS reactor. The immobilized enzymes are applied in 12 batch cycles of scFOS production reaction before regeneration of anion exchange resins, which is achieved by washing the resins with 4 bed volumes of 4% (w/v) NaOH solution.

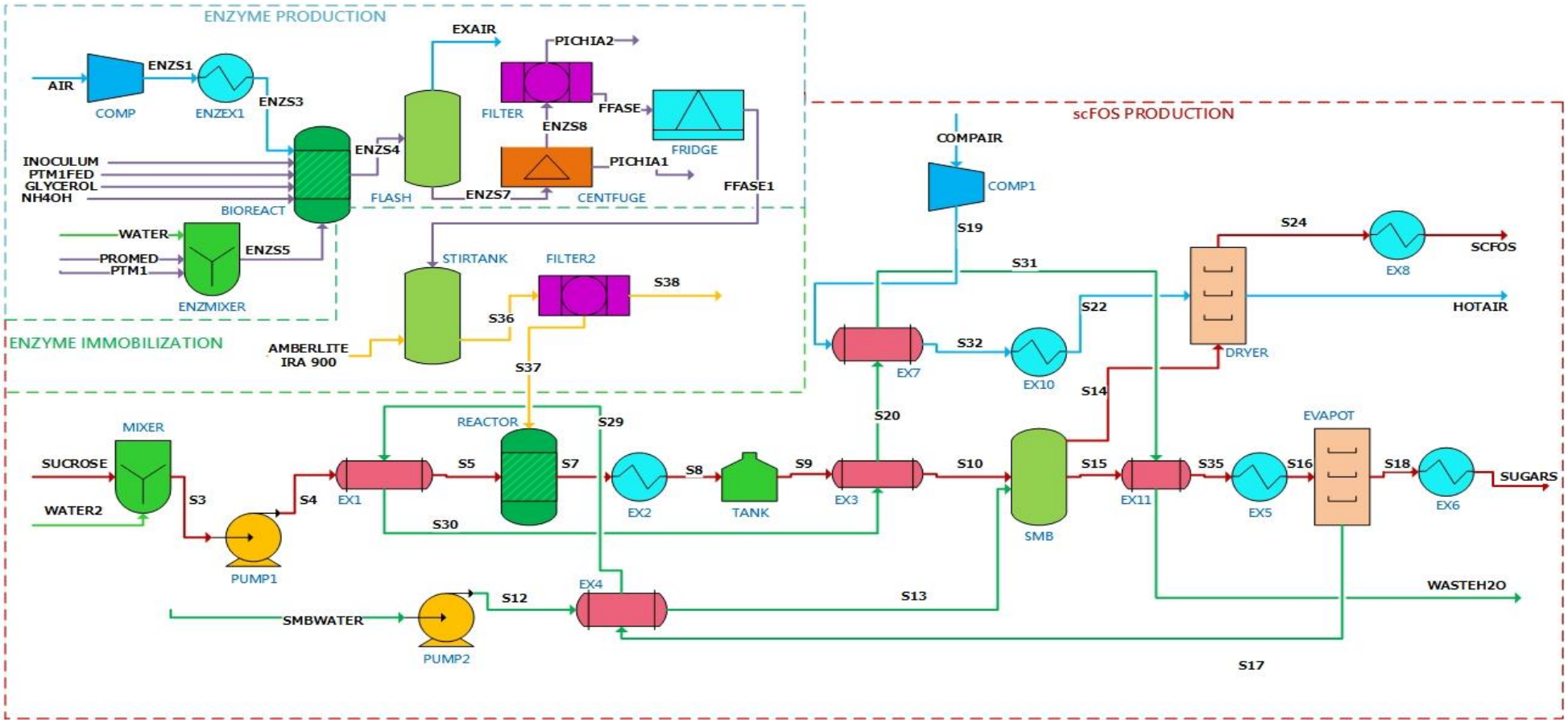

Figure 5.3: Process flow diagram for 2000 tpa scFOS producti on with the amberlite IRA 900 immobilized enzyme system

5.3 Results and discussions

5.3.1 Mass and energy balances

A summary of the mass and energy balances for the investigated scenarios is displayed in Table 5.2. The results obtained from experimental work recorded scFOS yields of 61%, 59% and 51% ($w/w_{total\ sugars}$) for FE, CAIE and AIE respectively [32]; the observed yields could be maintained for the CAIE and AIE methods for 6 and 12 recycles of reuse, respectively, before decreases were observed. Based on these results, significant reductions of 33% and 86% in the demand for the high-cost enzyme biocatalyst for the CAIE and AIE systems, respectively, were achieved. This is effectively a savings in operating and capital expenses, as smaller equipment capacity and lesser amounts of raw materials would be required to meet the enzyme demand in the case of the immobilized enzymes. On the contrary, 3.4% and 19.5% more sucrose is required by the CAIE and AIE systems, respectively, to meet the scFOS production target, due to their relatively lower scFOS yields compared to the FE system [32]. Generally, the conversion of sucrose in the scFOS reaction is about 90% indicating the presence of appreciable amounts of the unconverted sucrose [31], [32], [48]. Also present are the undesired end products of the scFOS reaction, glucose and fructose. The by-product sugars stream (a mixture of sucrose, glucose and fructose), which is somewhat sweeter than an equal mass of sucrose [49], can be sold to generate revenue after concentration in the evaporator. The AIE system generated the highest amount of by-product sugar of 1920 tpa due to the combined effect of low scFOS yield and high sucrose feedstock requirement. Followed by the AIE system was the CAIE and FE systems with 1390 tpa and 1280 tpa respectively. A detailed balance of components in all case scenarios is provided in the Appendix B.

Table 5.2: Summary of mass and energy balance of scenarios for production of 2000 tonnes per annum scFOS case scenarios

Parameter	**Unit**	**System**		
		FE[a]	**CAIE[b]**	**AIE[c]**
Enzyme supernatant demand	tpa	23.8	15.9	3.4
Number of recycles per enzyme immobilization		-	6	12
Raw material				
Sodium alginate powder	kg/yr	-	2528	-
Amberlite IRA 900	kg/yr	-	-	124.3
NaOH pellets	kg/yr	-	-	24.8
$CaCl_2.6H_2O$	tpa	-	27.7	-
Sucrose	tpa	3280	3390	3920
Product				
Short-chain fructooligosaccharides (scFOS)	tpa	2000	2000	2000
By-product sugars (sucrose, glucose and fructose)	tpa	1280	1390	1920
Utilities				
Electricity demand	kW	104.04	109.25	123.98
Heating demand	kW	426.48	448.54	510.10
Cooling demand	kW	55.04	54.64	56.96

[a] Free enzyme, [b] Calcium alginate immobilized enzyme, [c] Amberlite IRA 900 Immobilized enzyme

Furthermore, the introduction of the immobilization stage was accompanied by the need for additional raw materials specific to the applied immobilization technique. The AIE system required 124.3 kg/yr of the Amberlite IRA 900 anion exchange resin and 24.8 kg/yr of the NaOH pellets for regeneration of the ion exchange resins prior to re-use in subsequent immobilization. The CAIE also required 2527 kg/yr of sodium alginate powder and 27.7 tpa of $CaCl_2.6H_2O$ to meet the immobilized enzyme demand. This could translate into increased raw material cost in association with the immobilized enzyme systems if the savings on raw materials from the reduction in enzyme demand is unable to offset the additional cost of immobilization.

The AIE system required the most electricity of 123.98 KW followed by CAIE and FE systems with 109.25 kW and 104.04 kW respectively, chiefly due to the increased plant capacity of the scFOS production section to accommodate the increased feed rate of sucrose in the immobilized enzyme systems, hence the corresponding rise in utility consumption. Also additional utility requirement accompanied the immobilization section typically the power requirement for stirring in the AIE and CAIE systems and the power required to compress the alginate enzyme slurry through the small nozzles forming the beads in the CAIE system. The spray dryer in all three cases contributed to over 95% of the electricity demand of the entire process, because it requires a large amount of compressed hot air to rapidly dry the scFOS syrup, as it is fed into the drying chamber through the atomizer [50]. The electricity demand values are comparable to the 26.73, 21. 58 and 16 KW reported by Mussatto et al. (2015) [36] for 148.9, 158.3 and 232.6 tpa scFOS production capacities respectively.

The most heat intensive equipment were the spray dryer and the evaporator both operating at high temperatures. The steam supply to the evaporator and the spray dryer contributed about 46% and 43% respectively to the heating demands in all three scenarios as the product scFOS was required in powdered form and the by-product which could generate some revenue when concentrated. The highest steam consumption of 510.10 kW was associated with the AIE system followed by the CAIE and FE systems with 426.48 and 448.54 kW respectively which translates into 8,459, 7,438 and 7,072 tpa of steam for the AIE, CAIE and FE systems respectively. These are higher than that reported by Mussatto et al. (2015) [36] probably due to the inclusion of the spray dryer in our study.

The AIE recorded the highest cooling demand of 55.04 KW followed by the FE and CAIE systems with 54.64 and 56.96 kW respectively which translates into 97 323, 93 959 and 93 280 tpa of cooling water for the AIE, FE and CAIE systems respectively. Cooling water was required to maintain the temperature of the enzyme fermenter as a result of the heat generation associated with the growth of the media. Of all three systems, the fermenter of the FE system had the highest cooling demand of 8.43 kW since it had the highest capacity in correspondence to the highest enzyme demand [32]. The effluent from the scFOS production needed to be cooled to allow time for stabilization before being separated in the SMB. Also, the products from the spray dryer and evaporator (SCFOS and SUGARS in Figure 5.1) were

rapidly cooled to room temperature to minimize any chance of thermal decomposition. The cooling demand in the scFOS production segment accounted for 84.7% of the total cooling demand in all three scenarios.

5.3.2 Economic evaluation

5.3.2.1 Capital estimation

The mass and energy balances provided the basis from which the sizes of equipment were estimated, in order to determine the total equipment purchase cost (TEPC). The TEPC then formed the seed from which the capital investments were estimated. The total capital investment (TCI) estimation is provided in Table 5.3. By virtue of the decrease in the enzyme demand as a result of immobilization, there was a 22% and 68% reduction in the equipment cost of the enzyme production section for the CAIE and the AIE systems respectively compared to the FE system [32]. The enzyme production section accounted for about 11%, 9% and 3% of the TEPC in the FE, CAIE and AIE systems respectively, making it a minor contributor to the TEPC. But that saving was countered by the corresponding increase in the cost of equipment due to the required increase in equipment capacity to process the higher sucrose demand in the case of the immobilized enzymes because of relatively lower scFOS yields. This is evident in the 5% and 13% increase in the equipment cost of the scFOS production sections of the CAIE and AIE systems respectively compared to the free enzyme. Additional equipment costs were incurred by the inclusion of immobilization segment. The equipment costs of the immobilization segment of the CAIE and AIE systems amounted to $33 000 and $22 000 respectively. This translates into 1.4% and 0.9% of the TEPC for the CAIE and AIE systems respectively, which is virtually insignificant due to the simplicity of the selected immobilization processes [15], [32].

The spray dryer represented the highest equipment cost (68%, 68% and 73% of TEPC for FE, CAIE and AIE systems respectively) in all three cases. The total capital investments (TCI) amounted to 15.46, 15.95 and 16.19 million US$ (M$) for the FE, CAIE and AIE systems respectively. The working capital (WC) was estimated as 5% of the FCI and therefore followed the same trend as the TCI.

Table 5.3: Summary of capital estimation for case the studied scenarios using a modified costing sheet from Choi and Lee [45]

Item	FE system	CAIE system	AIE system
Equipment cost		**Cost (US $)**	
Enzyme production unit	251, 000	197,000	80,000
Enzyme immobilization unit	-	33,000	22,000
scFOS production unit	1,983,000	2,075,000	2,239,000
Total equipment purchase cost (TEPC)	**2,234,000**	**2,305,000**	**2,341,000**
Installation (70% of TEPC)	1,564,000	1,614,000	1,639,000
Process piping (35% of TEPC)	782,000	807,000	819,000
Instrumentation (40% of TEPC)	894,000	922,000	936,000
Insulation (3% of TEPC)	67,000	69,000	70,000
Electrical (10% of TEPC)	223,000	231,000	234,000
Buildings (45% of TEPC)	1,005,000	1,037,000	1,053,000
Yard improvement (15% of TEPC)	335,000	346,000	351,000
Auxiliary Facilities (40% of TEPC)	894,000	922,000	936,000
Total plant direct cost (TPDC)	**7,998,000**	**8,252,000**	**8,381,000**
Engineering (25% of TPDC)	1,999,000	2,063,000	2,095,000
Construction (35% of TPDC)	2,799,000	2,888,000	2,933,000
Total plant indirect cost (TPIC)	**4,799,000**	**4,951,000**	**5,028,000**
Contractor's fee (5% of [TPDC + TPIC])	640,000	660,000	670,000
Contingency (10% of [TPDC + TPIC])	1,280,000	1,320,000	1,341,000
Other costs (OTC)	**1,919,000**	**1,980,000**	**2,012,000**
Fixed capital investment (FCI)	**14,720,000**	**15,186,000**	**15,423,000**
Working capital (WC)	**736,000**	**759,000**	**771,000**
Total capital investment (TCI)	**15,456,000**	**15,946,000**	**16,194,000**

FE-Free enzyme, **CAIE**-Calcium alginate immobilized enzyme, **AIE**-Amberlite immobilized enzyme

5.3.2.2 Estimation of operating cost

The total operating cost (TOC) was estimated based on the mass and energy balance results obtained from the Aspen Plus simulation of the various scenarios. A summary of the TOC is presented in

Figure 5.4. The price of sucrose constituted the bulk (>90%) of the raw material cost, which was the greatest contribution to the variable operating cost (VOC) for all three scenarios. This is in line with that reported by Vaňková et al. (2008) [25], since the cost of sucrose constituted 82% of the raw material cost, which also constituted 90% of the operating cost. The raw material cost for AIE system was the highest at 1.95 million US$ per annum (M$/yr) due to the high sucrose demand followed by CAIE and FE systems at 1.50 M$/yr and 1.42 M$/yr respectively [32]. The saving on the raw material cost due to the reduced enzyme demand in the immobilized enzyme systems was minimal compared to the additional raw material cost incurred as a result of immobilization and increase in the sucrose demand.

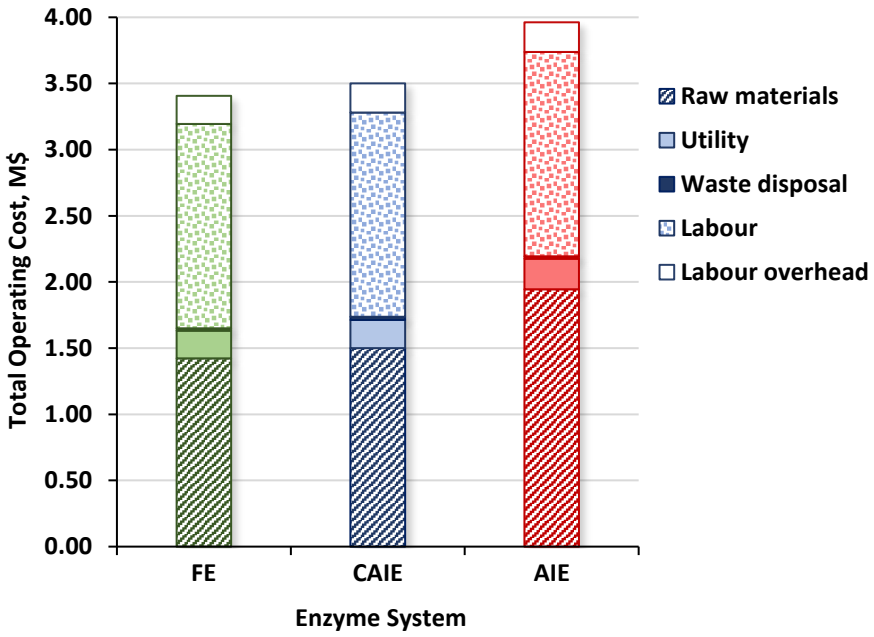

Figure 5.4: Estimation of total operating cost for case scenarios

The Utility cost was the second highest contributor, followed by the cost of waste disposal. The cost of waste disposal was estimated based on that reported by Dutta et al. (2015) [39], 5.9 and 33.5 $/ton for liquid and solid waste disposals respectively. Generated wastes included spent ion exchange resins, spent calcium alginate beads, yeast cells, spent NaOH

solution, spent $CaCl_2$ solution, and wastewater. Consequently, the AIE system recorded the highest VOC of approximately 2.19 M$/yr followed by the CAIE and FE systems at 1.74 M$/yr and 1.65 M$/yr respectively. The order of magnitude of the fixed operating cost (FOC) followed that of the fixed capital investment (FCI) since the maintenance cost, insurance and taxes were estimated as fractions of the ISBL and FCI respectively. Therefore, the highest FOC of 1.77 M$/yr was associated with the AIE since it also recorded the highest FCI of 15.42 M$. The total operating costs for the FE, CAIE and AIE systems were 3.40, 3.50 and 3.97 M$/yr respectively.

5.3.3 Profitability analysis

A discounted cash flow rate of return (DCFRR) analysis based on the real term discount rate of 9.7% was conducted using the TCI and TOC in all three scenarios to determine the minimum selling price (MSP). An assumed selling price of scFOS of 5 $/kg from that reported in literature was used as the standard in testing for viability [17]. The cost of consumables and selling prices were kept constant throughout the project life span of the plant hence the use of the 9.7% real term discount rate. Table 5.4 presents the minimum selling prices for all three scenarios. The MSPs for the case scenarios were estimated under two considerations: without the sale of the by-product sugar and with the sale of the by-product sugar. The price of by-product was pegged at 0.2 $/kg which is about 50% less than that reported in literature [25]. This was done to assume a worst-case scenario situation of the by-product sale, as there may be a situation where it would be sold for less than the stipulated value in literature as it is not a highly demanded product.

Table 5.4: Comparative summary of some economic parameters for production of powdered and syrup scFOS for case scenarios

Parameter	FE system		CAIE System		AIE system	
	Powder	**Syrup**	**Powder**	**Syrup**	**Powder**	**Syrup**
Product rate, tpa	2,000	4,300	2,000	4,300	2,000	4,300
Fixed capital investment, US $	14,800,000	4,700,000	15,200,000	4,900,000	15,500,000	4,300,000
Total utility cost, US $	209,000	132,000	213,000	132,000	231,000	139,000
Total operating cost, US $	3,410,000	3,260,000	3,510,000	3,350,000	3,970,000	3,800,000
Minimum selling price (without by-product sale), $/kg	2.77	1.97	2.85	2.03	3.10	2.21
Minimum selling price (with by-product sale), $/kg	2.61	1.82	2.69	1.87	2.94	2.06

FE-Free enzyme, **CAIE-**Calcium alginate immobilized enzyme, **AIE**-Amberlite immobilized enzyme

Without factoring in the sale of the by-product, the FE system recorded the lowest MSP of 2.77 $/kg, which makes it the most economically viable scenario chiefly due to it requiring the least amount of feed sucrose to meet the set production target [32]. This rippled out, affecting the TCI and TOC as the least of both parameters were associated with the FE system. The CAIE system was the second most viable with MSP of 2.85 $/kg followed by the AIE system with MSP of 3.10 $/kg. It is clear that the scFOS yield obtained with the biocatalyst plays a very significant role in the profitability of the scFOS production plant due to the impact of the sucrose demand [32]. The MSPs of all three scenarios were well below the set scFOS selling price of 5 $/kg (44.6%, 43% and 38% less for the FE, CAIE and AIE systems respectively) and therefore demonstrate significant economic viability.

A breakdown of the scFOS production cost revealed that the scFOS production segment contributed to over 80% of the total product cost for all of the production scenarios. This is because of the knock-on effect of the high tonnage of sucrose consumed in the scFOS production process. The costs associated with the enzyme production and enzyme immobilization were very minimal compared to that associated with the scFOS production step. Therefore, the savings on enzyme production by immobilization had little or no effect on the economics of the entire production plant. When the by-product sale was factored into the MSP calculation, the MSPs for the FE, CAIE and AIE systems decreased by 3.6%, 5.2% and 5.2% respectively. The order of economic viability was the same as when the by-product sale was not considered.

One way of reducing capital cost is by producing the purified scFOS in syrup form, which eliminates the use of the spray dryer. Economic evaluation conducted for syrup scFOS production (Table 5.4) revealed significant reductions of 68.2, 67.8 and 72.3% on the FCI of the FE, CAIE and AIE systems respectively. The total operating costs also experienced 4.4, 4.6 and 4.3% reductions for the FE, CAIE and AIE systems respectively. The MSPs without by-product sales also experienced 29% reductions for all three systems. It is therefore evident that the nature of the final scFOS product plays a significant role in the economics of the plant as the syrup scFOS production requires less capital and operating costs. However, the powdered scFOS offers numerous advantages as it satisfies all forms of applications by both industrial and individual consumers [36]. It also allows for easy handling and transportation

as well as cutting down on packaging, transportation and handling costs. Vaňková et al. (2008) [25] reported 3.8% reduction in the TCI when producing scFOS in syrup form. The TOC was reduced by 2.8% and 1.1% when using food grade and industrial grade sucrose respectively in syrup scFOS production. The cost of sucrose can therefore be influenced by obtaining from a supplier at a cheaper price or obtaining the sucrose by-product streams from other biorefineries. A potential feedstock worth exploring is sugarcane molasses (A-molasses) as it contains about 80% sucrose.

The effect of scFOS production capacity on MSP and FCI (Figure 5.5) was investigated by performing economic evaluations of simulated 5000 and 10000 tpa production capacities of the FE, CAIE and AIE systems. This was done to see if the effect of economy of scale could lead to the convergence in the MSPs of the case scenarios at a certain production target. The MSPs for the FE, CAIE and AIE systems experienced 10, 10 and 9% reductions respectively when the plant capacity was increased from 2000 to 5000 tpa. This also resulted in approximately 46% increase in the FCIs of all three enzyme systems. For a plant capacity of 10000 tpa, there was 16, 15 and 14% reductions in the MSPs of the FE, CAIE and AIE systems respectively compared to the 2000 tpa target. Conversely, the FCIs for the FE, CAIE and AIE systems increased by 204, 205 and 206% respectively. It is important to note that the order of profitability was maintained in all three production capacities. The FE was the most profitable, followed by the CAIE and AIE systems respectively.

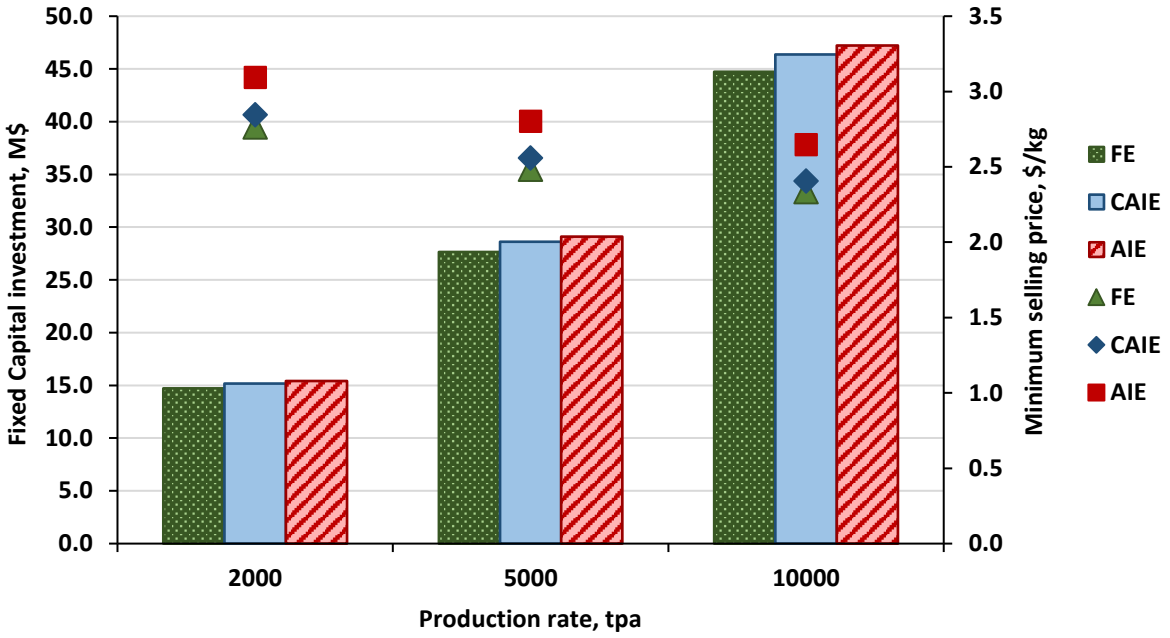

Figure 5.5: Fixed capital investments (bar charts) and minimum selling prices (scatter plots) for different scFOS production levels using the Free enzyme (FE) system, Calcium alginate immobilized enzyme (CAIE) system and Amberlite IRA 900 immobilized enzyme (AIE) system

5.3.4 Sensitivity analysis

In the sensitivity analysis, the effect of changes in TOC, FCI, %IRR, sucrose cost, Income tax, utility cost and WC on the MSPs of the three scenarios were investigated. The change in MSP was evaluated for a 20% increase and decrease in the selected economic parameters. As displayed in Figure 5.6, the parameters had almost identical effects on the MSPs in all three case scenarios. The sensitivity was only reported on the MSPs calculated without considering the by-product sale as similar effects was observed on the MSPs with by-product sale consideration. The TOC had the greatest effect on the MSPs in all three case scenarios followed by the FCI. The %IRR had the third greatest effect on the MSP followed by the sucrose cost for the FE and CAIE systems. The order was reversed in the AIE system most likely due to the high sucrose demand to meet the set production target. The parameters that had the least effects on the MSP were income tax, utility cost and working capital.

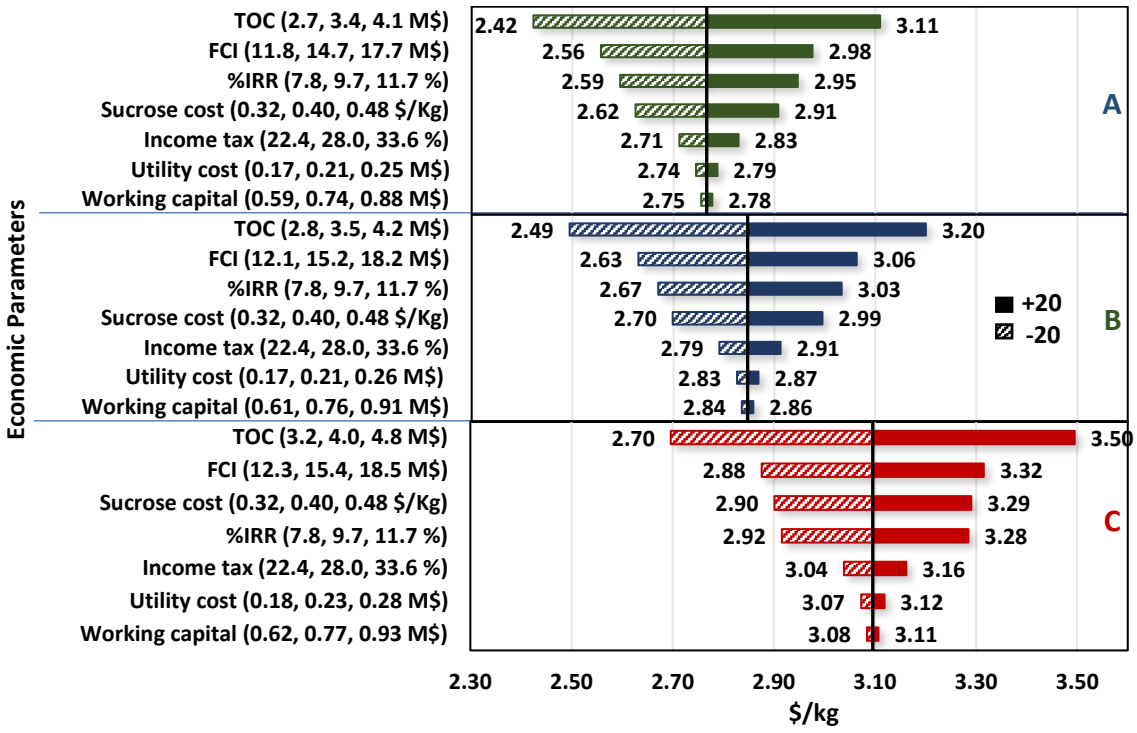

Figure 5.6: Economic sensitivity analysis of A-Free enzyme (FE) system, B-Calcium alginate immobilized enzyme (CAIE) system and C-Amberlite IRA 900 immobilized enzyme (AIE) system

5.4 Conclusions and future prospects

For the set production scale, all three scenarios were economically feasible as the calculated MSPs were well below the set target of 5 $/kg of scFOS. However, the FE system demonstrated the highest profitability by recording the lowest MSP of 1.82 $/kg compared to 1.87 and 2.06 $/kg for the CAIE and AIE systems respectively. The FE system is also the safer option considering that additional technical challenges may be associated with the operation of the immobilization unit. Even though the introduction of immobilization achieved some savings on the economics of the plant, it was outweighed by the additional costs incurred due to the limited reusability of immobilized enzymes and the low scFOS yields obtained. A future work worthy of consideration is to annex an scFOS production facility to a typical sugar mill to process the residual molasses into a more valuable product.

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Chapter 6

6 Optimization of inulooligosaccharides production from inulin-rich substrates extracted from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers in a biorefinery concept

Manuscript

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Short summary

Objective 5, which involved the optimization of IOS production from the various inulin-rich substrates obtained from JA tuber is addressed in this chapter (CHAPTER 6). This was conducted to achieve some process improvement on the process of IOS production from JA tubers in order to minimize the effective production costs (CHAPTER 7) for a more competitive techno-economic comparison with the scFOS production processes (CHAPTER 5). Three inulin-rich fractions (unprocessed JA powder, solid residue after protein extraction and inulin-rich extract) were obtained from the fresh JA tuber and applied in IOS production. The combinations of JA tuber pre-extractions with subsequent IOS isolation, each represent alternative biorefinery-type scenarios, to optimise value extraction from JA tubers, and thereby effectively minimise the production cost of IOS from JA. The experimental optimisation performed in the present chapter, provide the process descriptions required in Chapter 7 for the comparison of these scenarios, to identify the scenario preferred for low-cost IOS production. In the present chapter the hydrolysis conditions for each substrate were optimized for IOS production using RSM with CCD and compared with pure inulin from chicory. All three substrates produced impressive specific IOS yields with the inulin-rich extract yielding the highest IOS yield of 82.3% (w/w_{inulin}). One key finding was that the coextraction of IOS and protein in the biorefinery concept results in a significant deficit (37%)

on the overall yield of IOS from the biomass. The JA tuber substrate demonstrated good potential as a suitable alternative to chicory for IOS production.

Declaration by the candidate:

With regard to Chapter 6, pg. 121 - 150, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Planning of experiments, execution of experiments, interpretation of results and compilation of chapter	80

The following co-authors have contributed to Chapter 6, pg. 121 - 150:

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Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 6, pg. 121 - 150,
2. no other authors contributed to Chapter 6, pg. 121 - 150, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 6, pg. 121 - 150, of this dissertation.

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Optimization of inulooligosaccharides production from inulin substrates extracted from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers in a biorefinery concept

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Abstract

The increasing global demand for inulin and inulooligosaccharides (IOS) has necessitated the exploration of alternate sources of inulin. In this study, Jerusalem artichoke (JA) tuber was investigated as a potentially suitable source of inulin for industrial production of IOS. Three different inulin-rich substrates were prepared from the JA tuber, i.e. the JA powder prepared from the dried JA tuber slices, the solid residues of the JA powder after protein extraction, and an inulin-rich extract from the JA residue after protein extraction. The effect of reaction conditions on the IOS production from the JA powder was studied, followed by the optimization of the IOS yields from the inulin-rich substrates by response surface methodology. The results were further benchmarked against pure chicory inulin. Under the optimal conditions, IOS yield of 80.0% (w/w_{inulin}) was obtained from the JA powder, which was comparable to the 79.6% (w/w_{inulin}) obtained from the pure chicory inulin. Co-production of protein and IOS from the JA tuber in biorefinery concept resulted in at least 37% deficit in the overall IOS yield (g of IOS per 100 g of JA powder) relative to that obtained by direct hydrolysis of the JA powder. Direct IOS production from the solid residues after protein extraction prevents further 8% loss of IOS which occurs when deriving the inulin-rich extract from the solid residue for enzymatic hydrolysis. Nevertheless, the inulin-rich extract demonstrated the highest specific IOS yield of 82.3% (w/w_{inulin}) due to minimized interference from fibers and other organics. For all the inulin substrates, the optimal enzyme dosage was determined to be 14.8 U/g_{inulin} with the reaction times less than 6 h. The IOS composition varied with every individual substrate as a result of variations in the inulin profiles of the substrates. However, the collective composition of the degree of polymerization (DP) 3 and 4 components was above 72% for all substrates. With the results obtained here, the JA tuber exhibits good potential for commercial production of IOS with possible advantage of cheaper biomass and

improved productivity. The biorefinery scenarios are suitable for IOS and protein co-production, and the preferred scenario will be determined by economic analysis, rather than technical performance.

6.1 Introduction

Inulooligosaccharides (IOS) and short-chain fructooligosaccharides (scFOS) are short-chain fructose-containing oligosaccharides [1], which are beneficial as food ingredients due to their functional properties, such as enhancing the human immune system, improving adsorption of vitamins and minerals from the gastrointestinal tract, reducing phospholipids, triglycerides and cholesterol levels in the blood and suppression of constipation or diarrhoea [2]. As sweeteners which have low caloric content and are non-cariogenic, they are diabetic friendly and may be used to combat obesity [3].

Short-chain fructooligosaccharides is obtained from sucrose and constitutes more than 50% of the global market of these short-chain fructose-containing oligosaccharides. However, it is challenging to obtain an scFOS yield above 60% ($w/w_{sucrose}$) [4]. Inulooligosaccharides is produced by the selective and partial hydrolysis of inulin with the advantage of higher IOS yields of 70-90% (w/w_{inulin}) [5]–[7].

The definition of IOS varies based on the specified range of degree of polymerization (DP). Known definitions include DP 2-8, DP 2-7, DP 3-7 and DP 3-5 [8]–[13]. Degree of polymerization 2 contains sucrose (GF), which does not possess any prebiotic or functional properties typical to IOS [2]. Also, sweetness and prebiotic properties are inversely proportional to the oligosaccharide chain length [14], [15]. Therefore, a more appropriate definition of IOS would be DP 3-5, which was adopted in this work. The specified IOS DP range of 3-5 contains inulotriose (F3), inulotetraose (F4), inulopentaose (F5), 1-kestose (GF2), nystose (GF3), and 1^F-fructofuranosylnystose (GF4), in varying proportions.

The production of IOS from inulin involves the application of endoinulinase in isolation, or as a mixture of endo- and exoinulinase. The endoinulinase enzyme (2,1-β-D-fructan fructanohydrolase, EC, 3.2.1.7) cleaves the internal bonds of inulin producing the short-chain IOS intermediates with a degree of polymerization (DP) between 3 and 5 [4]. The exoinulinase

enzyme (β-D-fructan fructohydrolase, EC3.2.1.80) breaks off fructose from the non-reducing β-(2,1) ends resulting in short-chain IOS intermediates. The hydrolysis reaction involving exoinulinase, if not controlled, will completely reduce the inulin into glucose and fructose monomers [16], [17]. Therefore, exclusively endoinulinase is used in most cases when producing IOS.

Presently, chicory is the main source of inulin for commercial IOS production. However, Jerusalem artichoke (*Helianthus tuberosus* L.) possesses some characteristics that make it a suitable alternative source of inulin and consequently IOS [18]. The Jerusalem artichoke (JA) tuber has inulin contents (65 - 80% dry weight) similar to that of chicory (average of 68% dry weight), coupled with a significant amount of protein (15 - 16% dry weight) [19], [20]. The JA inulin could be better suited for IOS production, since only 48% of its inulin have a DP ≥ 9, compared to 71% in chicory, which may translate into shortened hydrolysis reaction time or better yield of IOS [21]. The ability of JA to resist pests and diseases, frost and drought coupled with its ability to grow on most soils with little fertilizer requirements, relieves it of geographical limitations and reduces cultivation expenses [2], [22], [23].

In this study, the potential of JA tuber as a sufficient biomass for IOS production was explored. Firstly, IOS production by direct enzymatic hydrolysis of the inulin in JA powder was characterized and optimized. The pre-extraction of protein prior to inulin extraction has been determined as the best configuration to maximise protein and inulin as products from the JA tuber in a biorefinery concept [24]. This study further tested the potential of JA tuber as a multiproduct biorefinery feedstock for protein and IOS co-production, rather than protein and inulin, by investigating IOS production from the solid residue after protein extraction. The option of obtaining IOS from the inulin-rich extract, obtained from the JA solid residue prior to enzymatic hydrolysis, was also studied to ascertain the effect of interference from the cellulosic fibres and other organics on the IOS yield. The significantly shortened reaction times, high IOS yields and low-cost of JA biomass are characteristics that improve the economic feasibility of industrial bioprocesses [25], [26]. To the authors' knowledge, no studies have been conducted to explore the avenues of maximizing IOS yields from JA tubers using endoinulinase.

6.2 Materials and methods

6.2.1 Materials

HPLC-grade standards (D-glucose, D-fructose, and D-sucrose) and sodium acetate were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). The fructooligosaccharide standards (1-kestose, nystose, and 1^F-fructofuranosylnystose) were purchased from Wako Chemicals GmbH (Neuss, Germany). The fructooligosaccharide standard inulotriose and pure chicory inulin were purchased from Megazyme (Ireland). HPLC-grade 50% NaOH solution was obtained from Fluka (Merck, Darmstadt, Germany). Novozyme® 960 commercial endoinulinase enzyme preparation was obtained from Novozymes (South Africa). Fresh JA tubers were purchased from Glen Agricultural College Bloemfontein and Mountain Herb Estate Pretoria, South Africa. JA tubers were washed under running water, peeled and sliced into approximately 2 mm thick pieces. The tuber slices were dried in the oven at 60 ^oC for 10 h. The dried slices were then milled into powder and stored at -18 ^oC. All other chemicals and reagents used were of analytical grade purity as purchased.

6.2.2 HPLC: High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Analyses for sugars was performed according to that described by Trollope et al. (2014) [27]. The quantifications of glucose, fructose, 1-kestose, nystose, inulotriose and 1^F-fructosylnystose were done by comparing the peak areas to those of external standards. Other fructans were quantified by extrapolation from the ratio of peak areas of the curves generated with the pure standards.

6.2.3 Enzyme activity assay

Endoinulinase activity was determined using the method described by Mutanda et al. (2008) [13] with some modifications. An aliquot (0.3 mL) of enzyme solution was reacted with 1.2 mL of 5% (w/v) of pure chicory inulin solution (prepared by dissolving 5 g of pure chicory inulin in 100 mL of 0.1 M citrate-phosphate buffer pH 6.0) at 60 ^oC for 1 hour. The reaction was terminated by placing the Eppendorf tubes containing the reaction mixtures on ice and immediately adding 91.5 µL of 35% (PCA) solution. An 82.5 µL of 7 N KOH solution was then added after ten minutes. The mixture was then centrifuged at 14000 rpm for two minutes. The clear supernatant was collected and syringe filtered through 0.225 µm membranes into

clean sterile Eppendorf tubes discarding the crystal pellets. The samples were analyzed by HPLC to estimate the activity of the enzyme. One unit of enzyme activity is defined as the amount of enzyme producing 1 µmol of fructose per minute under the above specified conditions.

6.2.4 Extraction of proteins from Jerusalem artichoke

The protein and inulin extraction processes were carried out as described by Maumela et al (2019) [20]. A weighted quantity (15 g) of Jerusalem artichoke powder was dissolved in 100 mL water and pH adjusted to 5.0 using 1M H_2SO_4. The solution was incubated at 25 ^oC for 1 h and 120 rpm. The suspension was centrifuged at 14000 rpm for ten minutes. The supernatant was collected and filtered through 0.45 µm membranes. The solid residue was also collected and dried at 60 ^oC for 10 hours. The dried solid residue was considered as substrate for IOS production.

6.2.5 Extraction of inulin from the Jerusalem artichoke

A weighted quantity (10 g) of Jerusalem artichoke powder residue, obtained after protein extraction, was dissolved in 100 mL of water and pH adjusted to 7.0 using 1M NaOH. The solution was incubated at 70 ^oC for 1 h and 120 RPM. The suspension was centrifuged at 14000 rpm for ten minutes. The supernatant was collected and filtered through 0.45 µm membranes. The collected inulin juice was processed into powdered form by freeze drying. The inulin-rich extract constituted a substrate for IOS production.

6.2.6 Total inulin determination

The total inulin content (I) of samples was determined by the Eq. (1).

$$I = k(F_{tot} - F_f) \quad (1)$$

Where F_{tot} and F_f are the total and free fructose contents respectively, while k is a correction factor of 0.995 adopted to account for the glucose content and the water loss as a result of hydrolysis [28], [29]. To estimate the free fructose content, 40 mg of powdered sample was dissolved in 10 mL of water and samples were drawn for HPLC analysis. The total fructose content was estimated by total hydrolysis of inulin, 40 mg solid sample was dissolved in 10 mL of water followed by addition of 0.5 mL of 1M H2SO4, the sample was incubated at 90 ^oC for 1 h. The reaction was neutralized by adding 75 uL of 7N KOH. Samples were then analyzed by HPLC. The average degree of polymerization, DP(av) was also estimated by Eq. (2) below.

$$DP(av) = \frac{F_i}{G_i} + 1 \quad (2)$$

Where F_i and G_i are the fructose and glucose respectively released from the inulin.

6.2.7 Enzymatic production of inulooligosaccharides by partial hydrolysis of inulin

Weighted amounts of inulin-rich substrates corresponding to 25, 50 and 75 g_{inulin}/L in 0.1M of citrate-phosphate buffers pH 5.0, 6.0 and 7.0. Varying enzyme dosages of 25, 50 and 75 U/g_{inulin} were added to the resulting inulin solutions. The reaction mixtures with total volumes of 50 mL in 250 mL Erlenmeyer flasks were incubated separately at 55, 60 and 65 ^{o}C and 120 rpm for 12 hours. Samples were drawn at 2-hour intervals for 12 hours and analyzed by HPLC.

6.2.8 Maximization of IOS production from various inulin substrates

The production of IOS was optimized by response surface methodology (RSM) with central composite design (CCD). To optimize the yields of IOS from the various inulin substrates, a set of reaction parameters were simultaneously varied. The experimental design and modeling analysis were carried out using Statistica for Windows software version 13.5. The parameters that were varied for the CCDs were time (2, 4 and 6 hours) X_1 and enzyme dosage (5, 15 and 25 U/g_{inulin}) X_2. The experimental runs are shown in Table S1 of Appendix C. The optimum yield was estimated by a second order (quadratic) model Eq. (3).

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 \quad (3)$$

Where Y is the response variable, β_0 is the intercept, β_i and β_j are coefficients for the linear effects, β_{ii} and β_{jj} are coefficients for the quadratic effects, β_{ij} is the coefficient for the interaction effect, X_i and X_j are the input variables.

6.2.9 Statistical analysis

All analyses were done in triplicates from three independent runs. Standard deviations have been provided where necessary. Statistical analysis of data was carried out by one-way and two-way analysis of variance (ANOVA) using Statistica for Windows software version 13.5 and differences were considered statistically significant when $p < 0.05$.

6.3 Results and discussion

6.3.1 Estimation of sugar and inulin content of the inulin-rich substrates from JA tuber

Three inulin-rich substrates were obtained from the JA tuber. The JA powder was prepared from the dried JA tuber slices. The solid residues which were obtained after protein extraction from the JA powder, and the inulin-rich extract obtained from the JA solid residue after protein extraction. Table 6.1 provides the free sugar and inulin contents, and DPs of the various inulin-rich substrates obtained from the JA tubers. The average inulin content of the JA powder was estimated at 65.73% (65.73 g of inulin/100 g of JA powder), which is well within the 62 to 75% range reported in literature [30]. The DP(av) of inulin in the JA powder was estimated as 13.07, which is comparable to the ranges (7.5 – 19.7) reported in literature [30], [31]. Collectively, the glucose, fructose and inulin amounted to 78.92% ($w/w_{dry\ weight}$) of the dry JA powder.

The inulin content of the solid residue was estimated at 49.33% ($w/w_{dry\ weight}$) of the solid residue. With respect to the starting raw material, only 70.1% (46.10 g of inulin/100 g of JA powder) of the initial inulin amount was retained in the solid residue. This was as a result of coextraction of some of the inulin during protein pre-extraction. This is consistent with the reported 25.3% of the inulin in JA tuber which was coextracted during pre-extraction of protein [24]. The DP(av) of inulin in the JA solid residue was estimated as 16.66, which was higher than that of the JA powder because of the coextraction of majority of the lower DP oligosaccharides during the protein extraction due to their relatively higher solubility resulting in the solid residue containing the higher DP inulin fractions [32].

Table 6.1: Free sugar and inulin content and average DP of the different inulin-rich substrates obtained from JA tuber

	%, Dry weight			
Substrates	**Inulin**	**Free glucose**	**Free fructose**	**Average DP**
JA powder	65.73	2.61	10.58	13.07
Solid residue after protein extraction	49.33	1.2	8.83	16.66
Inulin-rich extract	56.81	2.77	10.86	14.35

DP- Degree of polymerization

It has been established that almost identical inulin yields are obtained with or without pre-extraction of protein [24]. However, the protein contamination is minimized when inulin is extracted from the solid residues obtained after protein extraction from the JA powder [24]. Therefore, the inulin-rich extract in this study was extracted after protein pre-extraction. This allows for greater selectivity of the protein and inulin extraction steps in the biorefinery application of the JA tuber. The inulin-rich extract contained 56.81% (w/ $w_{dry\ weight}$) inulin, which is well within the 55 to 68.5% range reported by Maumela et al. (2019) [24] and even greater than reported elsewhere [33], [34]. Particularly, the inulin content of the extract from the JA tuber was even greater than the 51.20% reported for chicory using the conventional hot water extraction process as was used in this study [35]. With respect to the starting raw material (JA powder), only 56.25% (36.97 of inulin g/100 g of JA powder) of the initial inulin content was recovered in the inulin-rich extract. This was as a result of the losses during protein extraction (29.86% of total inulin in the JA powder) and the recalcitrant inulin which could not be extracted from the solid residue (13.89% of total inulin in the JA powder). The inulin in the inulin-rich extract had a DP(av) of 14.35. Collectively, the inulin, fructose and glucose amounted to 70.44% (w/$w_{dry\ weight}$) in the inulin-rich extract. Zhengyu at al. (2005) and Bekers et al. (2008) reported 71.8 % and 77.70% carbohydrate contents respectively of the inulin juices they extracted from JA tuber indicating the presence of other extractives [8], [34].

6.3.2 Effects of temperature, pH, substrate concentration and enzyme dosage on IOS production from JA tuber

The activity of the commercial endoinulinase isolated from *A. niger* was estimated as 266 U/mL. In order to study the effect of the different parameters on IOS production, the classical one factor at a time experimental run was conducted. The purpose of this was to determine some preferred conditions and also allow selection of factor ranges for the experimental runs used in the CCD. The range of parameters investigated were temperature (55, 60 and 65 ^{o}C), pH (5.0, 6.0 and 7.0), substrate concentration (25, 50 and 75 g_{inulin}/L) and enzyme dosage (25, 50 and 75 U/g_{inulin}). These ranges were selected based on reports from literature indicating maximal IOS yields within these ranges [4], [6], [8], [13]. Except for the varied parameter in each scenario, all other parameters were kept constant at 60 ^{o}C, pH 6.0, substrate concentration of 50 g_{inulin}/L, reaction time of 10 h and enzyme dosage of 50 U/g_{inulin}. The investigation was carried out on the JA powder as it constituted the base raw material from which the other inulin-rich substrates were obtained.

Throughout this manuscript, the IOS yield was estimated as a percentage of the starting mass of inulin in each specific substrate (JA powder, solid residue after protein extraction, inulin-rich extract and pure chicory inulin) which is consistent with the definitions in literature [6]–[8], [36]. As shown in Figure 6.1A, the maximum IOS yield of 57% (w/w_{inulin}) was obtained at 60 ^{o}C. The IOS production also seems to be favoured by the mildly acidic pH of 6.0 as seen in Figure 6.1B. These conditions are consistent with that reported by Mutanda et al. (2008) for this endoinulinase [13]. By comparison, most endoinulinases displayed their highest activities and consequently maximum IOS yields within the acidic pH region of 5.0 to 6.0 and temperature range of 50 ^{o}C to 60 ^{o}C [6], [37]–[39].

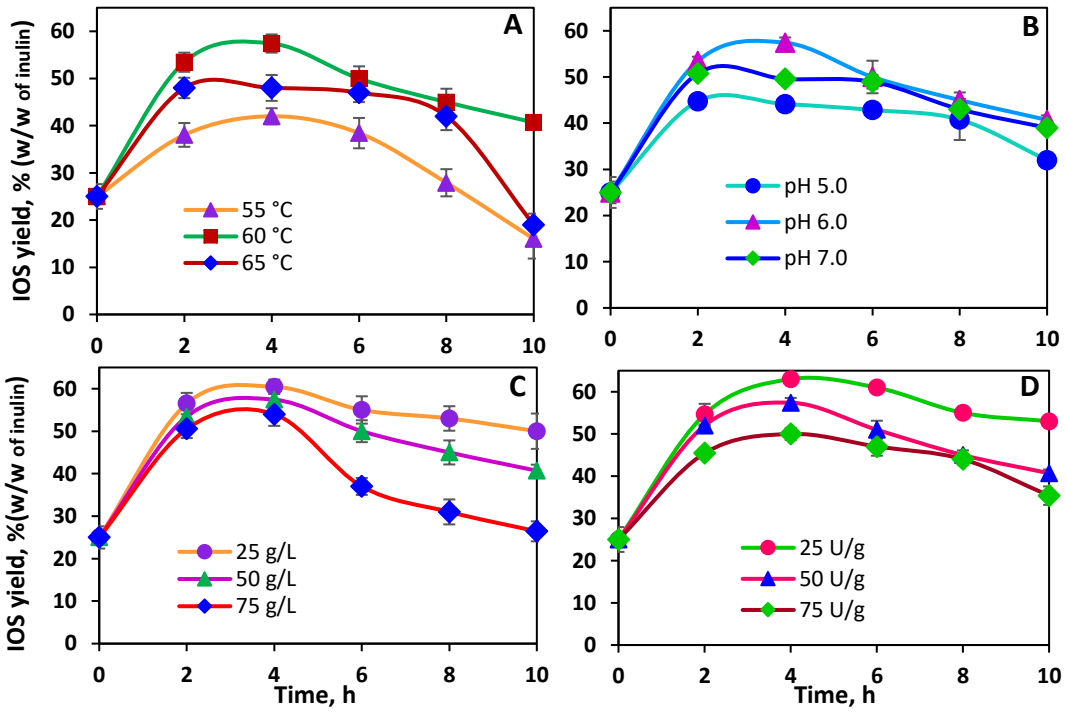

*Figure 6.1: Effects of Temperature **(A)**, pH **(B)**, substrate concentration **(C)** and enzyme dosage **(D)** on the production of IOS. Except for the varied conditions in each case, all other conditions were kept at pH 6.0, temperature of 60 °C, substrate concentration of 50 g_{inulin}/L and enzyme dosage of 50 U/g_{inulin}*

The 25 g_{inulin}/L substrate concentration (Figure 6.1C) produced the best profile with the highest IOS yield of 60% (w/w_{inulin}) after 4 h of reaction. This was followed by the 50 and 75 g_{inulin}/L substrate concentrations with 57 and 54% (w/w_{inulin}) respectively with significant difference ($p < 0.05$) between the yields from the various substrate concentrations. Increased viscosity accompanying the increased substrate concentration introduced some inhibition to the endoinulinase activity [8]. However, low substrate concentrations are not economically advisable due to the increased cost of concentration of product and increased size of process equipment. The substrate concentration of 50 g_{inulin}/L is advisable as it is a good balance between significant product yield, the economic implications of product dilution and a favorable solubility at the reaction temperature [6]. In other works, 50 g_{inulin}/L was reported as the optimal inulin substrate concentration for maximal IOS yields [8]–[10].

The 25 U/g of inulin enzyme dosage (Figure 6.1D) produced the highest IOS yield of 63% (w/w_{inulin}). With the enzyme dosage increased, lower IOS yields were obtained. With this result, it is anticipated that an even higher yield could be obtained with a lower enzyme

dosage hence the selection of 10, 15 and 20 U/g_{inulin} as the discrete levels of the enzyme dosage for the CCD experimental runs. In all four scenarios, it was observed that the yields increased rapidly and reached a maximum in less than 6 hours, after which there was a steady decline mainly due to further hydrolysis of the produced IOS.

6.3.3 Time course hydrolysis of inulin and variation of inulooligosaccharides composition

To investigate the variation in product composition during the enzymatic hydrolysis reaction, 50 mL of JA powder solution equivalent to 50 g_{inulin}/L (prepared by dissolving 4.71 g of JA powder in 50 mL of 0.1 M citrate-phosphate buffer pH 6.0) was exposed to an enzyme dosage of 50 U/g_{inulin} at pH 6.0 and 60 ^{o}C for 12 hours. Samples were drawn at 2-hour intervals and analysed by HPLC for IOS and other sugars. Figure 6.2 displays the yield and percentage composition of the IOS at the various time points. A comprehensive table of all the sugars and IOS components is provided in Table S2 of Appendix C.

In the graph of IOS yield and the IOS components of different DPs plotted as a function of reaction time (Figure 6.2): 25% (w/w) of the inulin present in the JA powder consisted of IOS at 0 h, of which F5 was the greatest amount of 9% (w/w_{inulin}). Since 52% of the JA inulin composition has DP ≤ 9 [21], it is expected that a significant fraction of that would constitute IOS. The inulin DP distribution in JA tubers varies depending on the factors such as cultivar type, species, harvest time, storage conditions and physiological age [30]. It is also expected that the initial IOS composition in the JA tuber would also vary depending on the above-mentioned factors [30], [40]. In another work, the initial IOS composition of JA powder was 20% (w/w_{inulin}) [8]. Cho et al. (2001) also reported a 30% (w/w_{inulin}) IOS content in a freshly extracted inulin-rich juice from chicory [36]. About 28.8% (w/w_{inulin}) initial IOS concentration was also was reported in crude chicory juice [41].

At 2 h (Figure 6.2), the amounts of DP3-DP5 all increased with F3 constituting the greatest fraction of 15.5% (w/w_{inulin}). At the same time, the GF4 and F5 reached their peak amounts of 11.1 and 8.9% (w/w_{inulin}) respectively. At 4 h (Figure 6.2), GF3 and F4 also attained their peak amounts of 8.5 and 13.2% (w/w_{inulin}). The GF2 and F3 amounts increased drastically within 4 h of reaction and maintained an almost constant concentration throughout the rest of the

reaction period. The nearly constant amounts of GF2, GF3 and F3 throughout the latter stages of the reaction shows the reluctance of endoinulinase to hydrolyse such species [4]. Rather, the enzyme is said to have a higher affinity towards the higher DP oligosaccharides considering the high rate of decline of DP > 4 oligosaccharides [10].

The maximum IOS yield of 57.5% (w/w_{inulin}) was recorded at 4 h, after which there was a steady decline in the IOS yield. At the end of the reaction period (12h), the IOS yield was 36% (w/w_{inulin}) of which F3 constituted the greatest percentage. The decline in IOS yield at the later stages of the reaction (Figure 6.2) indicated continued hydrolysis of the higher DP inulooligosaccharides (DP ≥ 4) into mono- and disaccharides signifying the presence of exoinulinase activity. The effect of the exoinulinase activity can also be seen in the significantly high fructose component (34.6% [w/w_{inulin}]) obtained at the end of the reaction period (12 h) (Table S2 of Appendix C). The autogenous exoinulinase activity in the JA powder [8] may have caused the decline in the IOS yield after 4h. The reaction therefore, needs to be truncated at an earlier time if a high IOS yields is desired.

The significantly high F3 amounts obtained during the initial stages of the reaction suggested that the majority of the higher DP oligosaccharides were cleaved in such a way to produce F3. This is a characteristic mechanism of many endoinulinases of *Aspergillus* origin [42], [43]. Chen et al. (2012) reported F3 and F4 as the major components of IOS produced using endoinulinase from *Aspergillus ficuum* [44]. Another author also reported DP3 and DP4 as the main components of the IOS produced using endoinulinase from *Aspergillus fumigatus* [37]. Endoinulinase from *Aspergillus arachidicola* also produced IOS with DP3 and DP4 constituting the greater fraction [6].

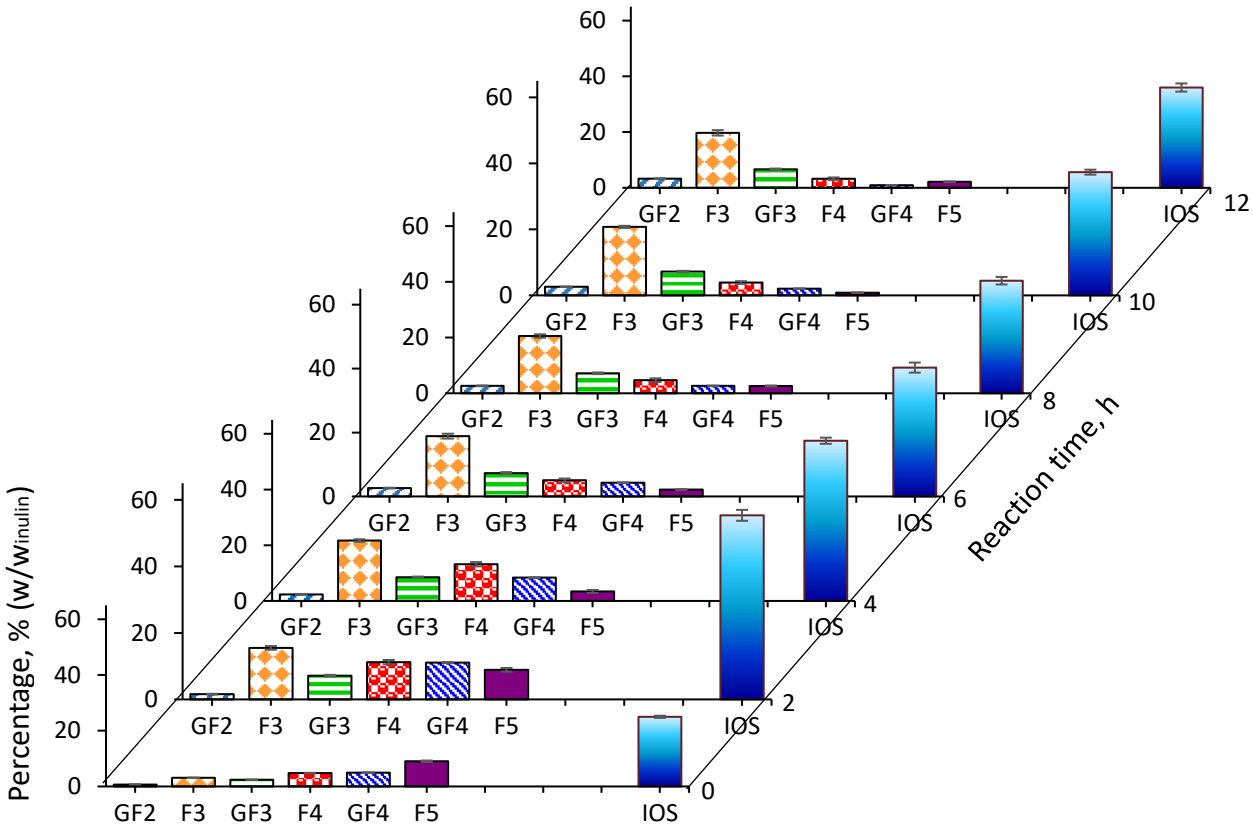

Figure 6.2: Bar chart presentation of the percentages of the IOS components and IOS yield during enzymatic hydrolysis on JA powder. Reaction condition: 50 g_{inulin}/L, 50 U/g_{inulin}, pH 6.0, 60 °C and 12 hours. The IOS yield was determined by the summation of the percentages of the individual IOS components

6.3.4 Optimization of inulooligosaccharides production from JA powder

From the combination of preliminary experiments, the preferred pH, temperature and substrate concentration were estimated as 6.0, 60 °C and 50 g_{inulin}/L respectively. These were kept constant while varying the only the reaction time (2, 4 and 6 h) X_1 and enzyme dosage (10, 15 and 25 U/g_{inulin}) X_2 for the CCD. The CCD design was conducted on the JA powder to ascertain the conditions for optimal IOS yield via direct hydrolysis of the powder. Table S4 of Appendix C shows the sugar and IOS component distributions for the 12 CCD runs. Due to the presence of inherent hydrolytic activity from exoinulinase synthesized in the JA tubers during growth, the GF2 amounts in the runs obtained from the hydrolysis of the JA powder were high, irrespective of the enzyme dosage [8].

The response surface for the CCD run is displayed in Figure 6.3A. Reaction time and enzyme dosage of 5.4 h and 14.8 U/g_{inulin} were predicted by the regression model Eq. (4) as the conditions for the optimal IOS yield of 80.0% (w/w_{inulin}) from the JA powder. The R^2 of the model was calculated as 0.96, which demonstrates sufficiency to fit the experimental data.

$$Y = 30.58 + 11.05X_1 + 2.88X_2 - 0.90X_1^2 - 0.07X_2^2 - 0.15X_1X_2 \quad (4)$$

The CCD run was also conducted on pure chicory [DP(av) = 27.5] to provide a benchmark for the yield obtained from the JA powder. Table S3 of Appendix C displays the distribution of the sugars and IOS components for the 12 CCD runs using the pure chicory inulin as substrate. The response surface from the CCD is displayed in Figure 6.3D. Significant quantities of F6 and GF6 were detected in majority of the runs with the pure chicory inulin, this can be associated with the high DP(av) of the pure chicory inulin compared to that of the substrates obtained from the JA tubers. The low percentage of the fructose (Table S3 of Appendix C) in the reaction mixture for the various runs with the pure chicory inulin indicates that the exoinulinase activity in the commercial enzyme is very minimal, as the fructose amounts obtained (0.7 – 3.4%) are even lower than those obtained in literature (2.4 – 10.2%) for partially purified endoinulinase enzymes and comparable to that obtained for the purified enzymes [7], [41], [45], [46]. The high concentrations of fructose (1.1 – 14.0%) obtained in the CCD runs on the JA powder relative to that of the pure chicory inulin can be attributed mainly to the exoinulinase activity inherent in the JA tuber [8]. The conditions for the optimal IOS yield of 79.6% (w/w_{inulin}) from pure chicory inulin as predicted by the fitted response surface regression equation in Eq. (5) were reaction time of 4 h and enzyme dosage of 14.8 U/g_{inulin}. The fitting value termed R^2 for the regression model was calculated as 0.90. Which demonstrated a good correlation between the experimental data and the predicted model.

$$Y = 39.20 + 11.61X_1 + 1.70X_2 - 1.36X_1^2 - 0.06X_2^2 + 0.05X_1X_2 \quad (5)$$

Table 6.2 provides a summary of the ANOVA for the CCD models of the various inulin-rich substrates. For both the JA powder and inulin extract, the time had the greater effect on the IOS yield than the enzyme dosage, which is confirmed by the higher degree of curvature observed on the plot of time against IOS yield (Figure 6.3). The negative signs on the quadratic terms of time and enzyme dosage (Eq 4 and 5) demonstrated a decrease in IOS when both factors were increased beyond a certain threshold. The surface plots portray similar trends

between the time and enzyme dosage and the IOS yields for both substrates. Increases in time and enzyme dosage resulted in corresponding increase in IOS until a threshold beyond which further increases adversely affected the IOS yield. The increasing yield can be attributed to sufficient enzyme-substrate contact time and increased hydrolytic activity as a result of increased enzyme dosage. The optimal reaction time for IOS production from the JA powder was longer compared to that of the pure chicory inulin. A possible reason could be the substrate inhibition as a result of interference from fibres and other organics in the JA powder as the powder is only 65% inulin. Nevertheless, the predicted yield from the JA powder is comparable to that from the pure chicory inulin.

Table 6.2: Analysis of variance for the CCD models for IOS production from the various inulin-rich substrates. ANOVA was determined with a 95% confidence level

	JA Powder		**Inulin-rich extract**		**Solid residue after protein extraction**		**Pure chicory inulin**	
Parameter	Sum of squares	p-value	Sum of squares	p-value	Sum of squares	p-value	Sum of squares	p-value
Linear								
Time (X_1)	93.25	0.024	6.87	0.411	13.30	0.170	63.91	0.026
Enzyme dosage (X_2)	45.24	0.048	7.82	0.384	18.22	0.133	41.30	0.045
Quadratic								
X_1^2	71.23	0.031	13.08	0.281	31.34	0.084	189.69	0.006
X_2^2	250.41	0.009	149.20	0.021	877.58	0.003	185.64	0.006
Interaction								
X_1X_2	36.00	0.059	0.00	1.000	42.25	0.064	3.28	0.420
Lack of Fit	93.84	0.070	111.64	0.112	447.32	0.080	51.98	0.121
Pure Error	4.67		22.75		6.00		11.28	

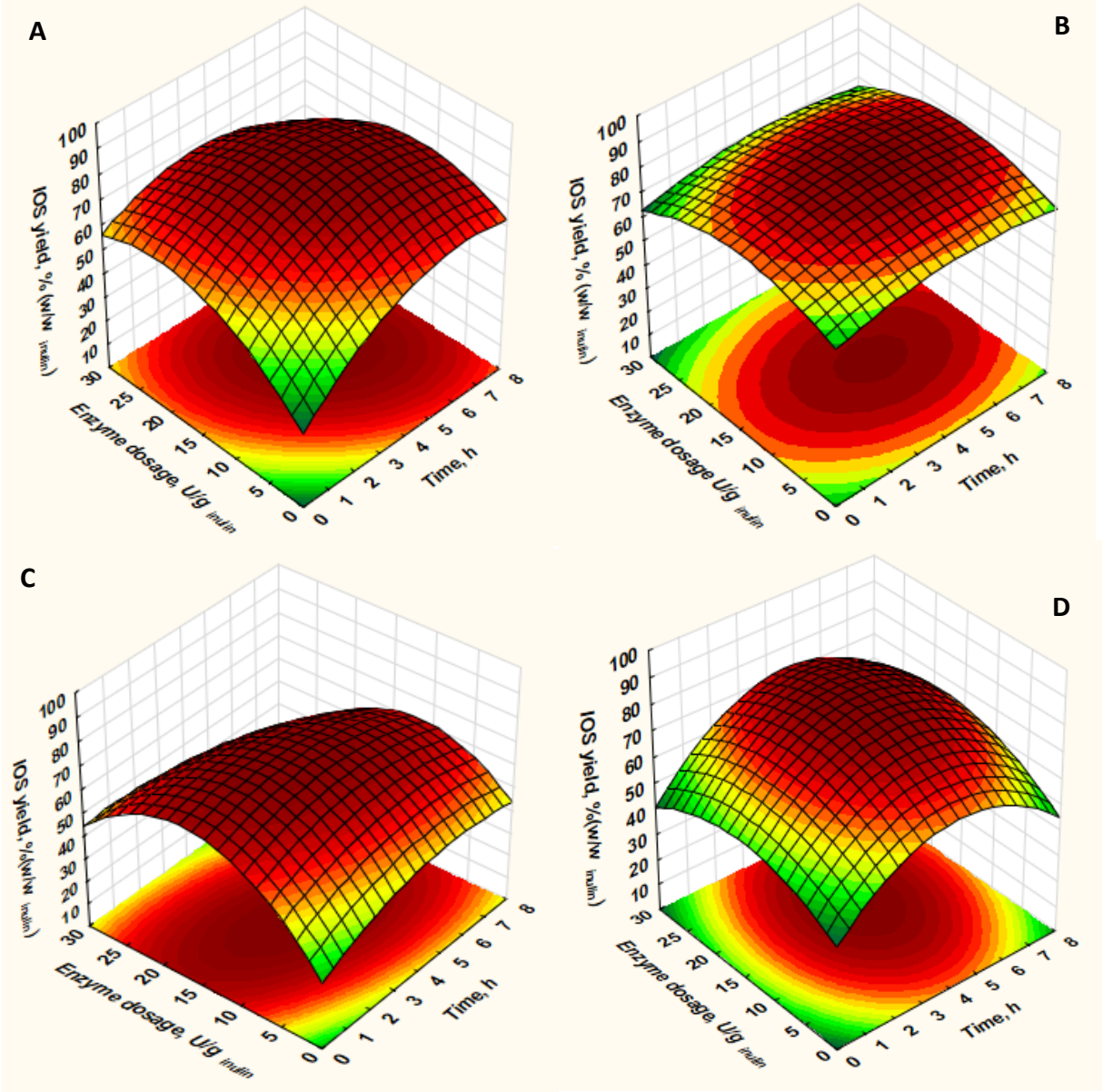

*Figure 6.3: Response surface for IOS production from **A**-JA powder, **B**-Inulin-rich extract from JA tuber, **C**-Solid residue after protein extraction and **D**-Pure chicory inulin*

6.3.5 Effect of inulin extraction on IOS production from JA powder in a biorefinery concept

In the quest to optimize the production of IOS from the solid residue after protein extraction in a biorefinery concept, consideration was given to the extraction of inulin from the solid residue prior to enzymatic hydrolysis. This may offer the advantage of eliminating the interfering proteins and fibers in the JA solid residue which may hinder enzymatic activity.

The CCD run was therefore conducted on the inulin-rich extract obtained from the JA residues after protein extraction to maximize the IOS yield from the extract. From the sugar and IOS component distribution data displayed in Table S6 of Appendix C, no GF6 and F6 amounts were detected in the hydrolyzed samples and relatively low GF5 amounts were also detected compared to that obtained with the JA powder. This is most likely due to the increased endoinulinase activity as a result of absence of interactions from fibres.

Figure 6.3B provides the surface response for the regression model Eq. (6). Similar to the pure chicory inulin, the predicted reaction time and enzyme dosage for the optimal yield of 82.3% (w/w_{inulin}) were 4 h and 14.8 U/g_{inulin} respectively. The fitted term R^2 for the model was 0.84, which implies that the model is a sufficient fit for the experimental data. In comparison to the JA powder, the inulin-rich extract obviously gives the better IOS yield as the optimal substrate. However, the IOS production by direct hydrolysis of the JA powder becomes significantly superior when the overall IOS yield is estimated, which is the IOS mass percentage of the starting raw material (JA powder) i.e. 52.6% and 30.42% for the IOS directly from JA powder and from the inulin-rich extract respectively. This is as a result of the inulin losses associated with the preparation of the inulin-rich extract.

$$Y = 65.20 + 3.32X_1 + 1.38X_2 - 0.36X_1^2 - 0.05X_2^2 \quad (6)$$

6.3.6 Effect of protein extraction on IOS production from JA powder in a biorefinery concept

The other alternative of direct enzymatic hydrolysis of the JA solid residues after protein extraction was also investigated. The solid residue obtained after protein extraction was subjected to a CCD experimental run to ascertain if the protein extraction has a significant effect on the IOS yield obtained from the JA tuber biomass. The sugar and IOS component distributions from the various runs are displayed in Table S5 of Appendix C. There seemed to be an increase in exoinulinase activity in the solid residues after protein extraction, which can be seen in the significantly high fructose components in the various runs.

The response surface diagram (Figure 6.3C) provides adequate visualization of the combined effect of the time and enzyme dosage on the IOS yield obtained. The time and enzyme dosage values for the maximal IOS yield of 72.0% (w/w_{inulin}) from the solid residue were predicted by

the regression model Eq. (7) as 4 h and 14.8 U/g_{inulin} respectively. The fitted term R^2 of 0.97 indicates a satisfactory correlation between the fitted and experimental data.

$$Y = 29.10 + 6.50X_1 + 4.31X_2 - 0.59X_1^2 - 0.13X_2^2 - 0.16X_1X_2 \quad (7)$$

The IOS yield obtained with the JA solid residue after protein extraction was significantly lower than that obtained from the direct hydrolysis of the JA powder as a result of increased interference since more biomass was required to meet the substrate concentration of 50 g_{inuln}/L due to the lower inulin content (49.33%) of the residue compared to the JA powder (65.73%). Another contributing factor was the increased exoinulinase activity in the JA solid residue as seen in the high fructose content (12.4 – 32.3%) of the CCD runs. In terms of the overall IOS yield with respect to the initial mass of JA powder, the solid residue produced an overall IOS yield of 33.20% which was significantly lower than that obtained for the JA powder. This was as a result of the inulin losses during protein pre-extraction. Nevertheless, this overall IOS yield was greater than that obtained for the inulin-rich extract.

Therefore, in the case of protein and IOS co-production from the JA tuber in a biorefinery concept, it may be better to produce the IOS directly from the solid residues after protein extraction instead of obtaining the inulin-rich extract from the residue prior to hydrolysis. This prevents any further losses associated with the additional extraction step. The use of the solid residue for IOS production provides the option of co-production of protein and IOS from the JA tuber in a biorefinery concept. However, this can only be acceptable if the economic benefits of the proteins extracted outweighs the inulin losses incurred. In a case where the protein extraction is not desired, IOS can be produced by direct hydrolysis of the JA powder.

6.3.7 Validation of optimal conditions and comparison of IOS composition of inulin-rich substrates

A validation experiment was conducted to confirm the predicted optimal conditions for maximum IOS yield from the various substrates. Table 6.3 provides a summary of the validation results. The predicted optimal IOS yields from the chicory inulin, JA powder, JA solid residue and the JA inulin-rich extract were 79.6, 80.0, 72.0 and 82.3% (w/w_{inulin}) respectively. The experimental yields obtained at the optimal conditions for the chicory inulin, JA powder, JA solid residue and the JA inulin-rich extract were 79.2, 76.3, 71.0 and 77.1 %(w/w_{inulin})

respectively. With these confirmatory runs, the model accuracies for the chicory inulin, JA powder, JA solid residue and the JA inulin extract were of 99.5, 95.2, 98.6 and 93.3% respectively.

The IOS composition from the optimal yields (Figure 6.4) were specific to each substrate mainly due to the variation in the DP of inulin each substrate. Nevertheless, DP3 and DP4 dominated the IOS composition for all substrates (above 72%). The IOS compositions from the various inulin-rich substrates suggest that IOS from the JA powder would be the sweetest due to the high percentage of the DP 3 and 4 components (80.3% [w/w_{IOS}]). Sweetness is a desired property alongside the prebiotic effects of the IOS as the IOS find significant application as a functional sweetener [47]. Similar to sweetness, the bifidogenic properties are also favoured by the lower DP oligosaccharides [12].

Table 6.3: Validation of optimal conditions for IOS production from the various inulin substrates

Substrate	Optimal conditions		IOS yield, % (w/w_{inulin})					Overall IOS yield[a], %
	Time, h	Enzyme dosage (U/g_{inulin})	Actual	Stdev	Predicted	Stdev	Model accuracy, %	
JA powder	5.4	14.8	76.3	1.5	80.0	4.0	95.2	52.6
Inulin-rich extract	4.0	14.8	77.1	3.5	82.3	4.1	93.3	30.4
Solid residue after protein extraction	4.0	14.8	71.0	2.9	72.0	3.6	98.6	33.2
Pure chicory inulin	4.0	14.8	79.2	1.0	79.6	4.0	99.5	*

The reaction temperature, pH and substrate concentrations were maintained at 60 °C, 6.0 and 50 g_{inulin}/L respectively for all substrates. Stdev - Standard deviation, [a] - IOS percentage of the initial mass of JA powder, *- not available

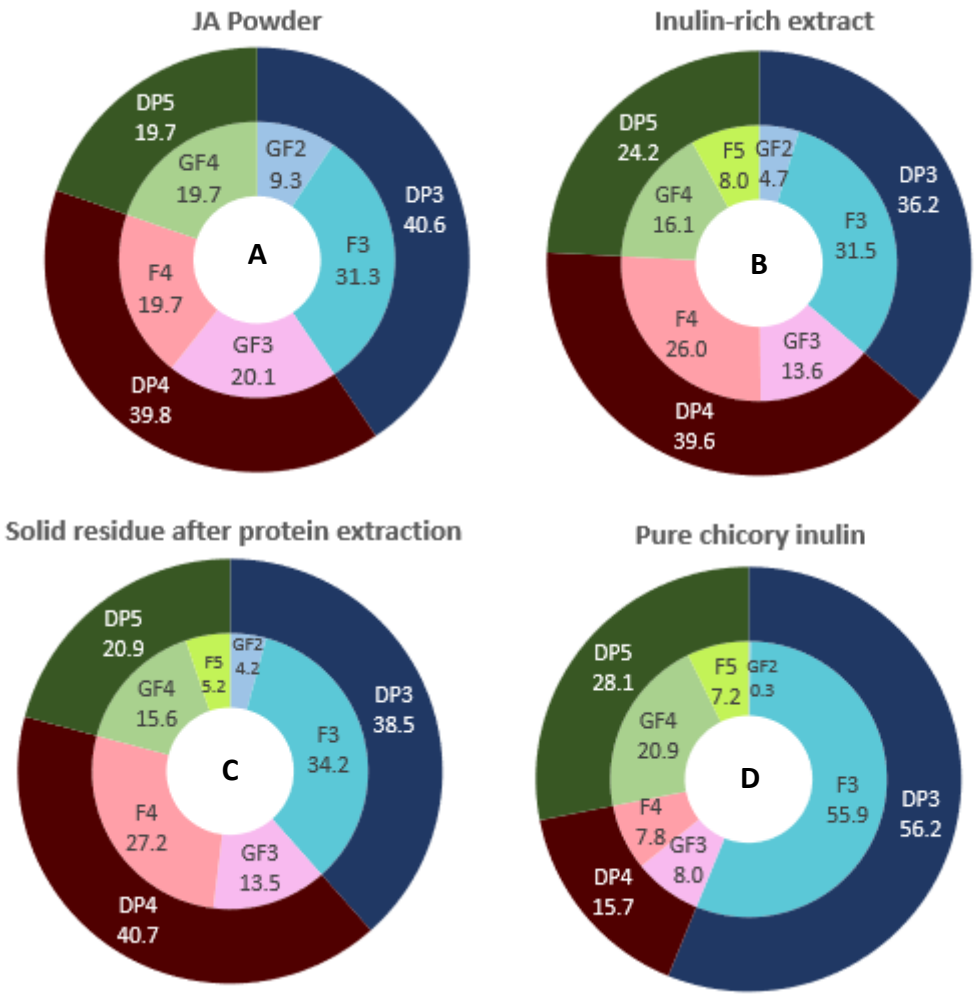

*Figure 6.4: Percentage composition (w/w$_{IOS}$) of IOS obtained from the inulin substrates under optimal conditions. **A**-JA powder, **B**-Inulin-rich extract from JA tuber, **C**-Solid residue after protein extraction and **D**-Pure chicory inulin*

The specific IOS yields obtained in this work are comparable to those obtained in literature (Table 6.4) for various inulin substrates. It must be noted that the IOS definition by DP range varies from one author to the other which introduces some form of bias in the yields reported to give an indication of high IOS yield. The reaction times (between 4-6 h) obtained for optimal IOS yields in this work are significantly shorter than those in literature (between 30 -72 h) [4], [11], [48]. A short reaction time is more desirable for industrial processes as it improves productivity.

Table 6.4: Inulooligosaccharides yields and DP ranges obtained in this study in comparison to other reports

Substrate	IOS yield, % (w/w)	Dp range	Reference
Pure Inulin	70.37	2-8	[8]
	91.66	2-7	[9]
	91.41	2-7	[9]
	83.29	2-6	[4]
	75.6	2-7	[10]
	71.2	3-7	[11]
	83	3-7	[12]
	72	3-7	[12]
	54	3-5	[13]
	79.2	3-5	This work
JA Powder	41.72	2-8	[8]
	76.3	3-5	This work
JA juice	79.8	2-8	[8]
Solid residue after protein extraction	71.0	3-5	This work
Inulin-rich extract from JA	77.1	3-5	This work

6.4 Conclusion

The production of IOS from the various inulin-rich substrates obtainable from JA tubers was investigated. The actual IOS yield of 80.0% (w/w_{inulin}) obtained by direct hydrolysis of the JA powder was comparable to the 79.6% (w/w_{inulin}) obtained from pure chicory inulin, which makes the JA tuber a suitable source of inulin for industrial IOS production with the added advantage of low cultivation demands. The inulin-rich extract appears as the optimal substrate for IOS production with an improved IOS yield of 3%. However, this resulted in a 42% potential reduction in the overall IOS yield as a result of inulin losses associated with the extraction steps. The pre-extraction of protein produced a 36% deficit on the overall IOS yield relative to direct hydrolysis of the JA powder. Therefore, in the biorefinery concept, the IOS production from the solid residues after protein extraction may be preferred over the inulin-

rich extract. IOS and protein can be produced from the JA tuber in a biorefinery concept, but at the expense of IOS yield per mass of JA powder. This work provides a reference point for an economic analysis to determine if protein extraction for application as nutraceuticals is beneficial enough to offset the associated IOS losses. The significantly shortened reaction times provide potential for improved productivity of commercial IOS production.

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Chapter 7

7 Techno-economic analysis of inulooligosaccharides, protein and bioenergy co-production from Jerusalem artichoke tubers in a biorefinery concept

Manuscript

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Short summary

The objective 6, which involves the techno-economic evaluation of IOS, protein, animal feed and bioenergy production from JA tubers in a biorefinery concept is outlined in this chapter (CHAPTER 7). Five biorefinery scenarios of IOS production from JA tubers were simulated in Aspen Plus® software using the experimental data for IOS production in CHAPTEER 6 and evaluated for economic feasibility. Only scenarios A and B demonstrated some margin of profitability, with scenario B being the more profitable. The key findings in this chapter was that, at the set IOS production scale, bioenergy production from the residues rather reduced the economic feasibility of the biorefinery as the additional capital and operating expenditure associated with the bioenergy production far outweighed the revenue generated. It was also discovered that the coproduction of protein introduced some economic deficit on the feasibility of the biorefineries. The best-performing scenario, which essentially gave the lowest value of MSP, was compared to the best case of the scFOS from sucrose scenarios to determine which product has the superior profitability (CHAPTER 5).

Declaration by the candidate:

With regard to Chapter 7, pg. 151- 194, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Simulation work, interpretation of results and compilation of chapter	80

The following co-authors have contributed to Chapter 7, pg. 151- 194:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
J.F. Görgens	jgorgens@sun.ac.za	General discussions and revision of chapter	10
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Signature of candidate:……

Date:……..

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 7, pg. 151- 194,
2. no other authors contributed to Chapter 7, pg. 151- 194, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 7, pg. 151- 194, of this dissertation.

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Techno-economic analysis of inulooligosaccharides, protein and biofuel co-production from Jerusalem artichoke tubers in a biorefinery concept

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Abstract

Jerusalem artichoke (JA) is a crop with great potential for application in a biorefinery. It can resist drought, pests and diseases and thrive well in marginal lands with little fertilizer application. The JA tubers contain considerable quantities of inulin, which is suitable for the production of IOS, as a high-value prebiotic, dietary fibre. In this study, five JA tuber biorefinery scenarios were simulated in Aspen Plus® and further evaluated by techno-economic and sensitivity analysis. Production of inulooligosaccharides (IOS), proteins and animal feed were studied in scenarios A and C, applying the various biorefinery configurations. Scenario B explored the production of only IOS with the sale of residues as animal feed, whereas Scenarios D and E investigated the coproduction of biogas and ethanol respectively, from the residues after IOS and protein production. Based on the economic indicators, the most favorable biorefinery configuration was scenario B which resulted the least MSP of 3.91 $/kg with correspondingly reduced total capital investment (FCI) and total operating cost (TOC) of 37.82 and 5.18 million US$ respectively. For the set production scale, it is more profitable when the residues are sold as animal feed instead of conversion into bioenergy due to the capital-intensive nature of the bioenergy production processes. The coproduction of protein rather had a negative impact on the economics of the IOS production process as the associated expenditure outweighed the associated revenue. Through the sensitivity analysis, it was discovered that the MSP is greatly dependent on the FCI and the internal rate of return (IRR). The best case of the JA tuber biorefineries (scenario B) was less profitable compared to the best case of the counterpart scFOS production from sucrose.

7.1 Introduction

Inulooligosaccharides (IOS) is a high value product which finds application as dietary component of foods, sweeteners and as pharmaceuticals [1]. As a functional food ingredient, IOS stimulates the human immune system, increases the adsorption of minerals and vitamins, reduces triglycerides and phospholipids levels in the blood, reduce the emergence of colon cancer [2]. As a prebiotic, IOS stimulates the growth of bifidobacteria in the colon and relieves constipation. It is also used to substitute fat, sugar and flour in dairy products, cereals and baked foods respectively to achieve low calorie content [3]. As low-calorie sweeteners which are non-cariogenic, IOS is diabetic friendly and may not cause obesity [4].

Inulooligosaccharides together with short-chain fructooligosaccharides (scFOS) constitute a significant fraction of the worlds prebiotic market which was estimated as 167 000 tonnes and 360 million Euros in 2014 [4]. Due to the increasing health awareness and increased consumption of functional foods, the IOS market has experienced an estimated annual growth rate of 15% [5].

Industrial production of IOS is carried out by the controlled or partial hydrolysis of inulin using an endoinulinase enzyme [14]. Chicory roots and Jerusalem artichoke (JA) tubers both contain significant levels of inulin (above 65% dry weight) [6]. Presently the major source of commercial IOS is the chicory inulin, probably because the complete agricultural package of breeding, production, harvest and processing of chicory is currently operational [6]. Also, the chicory inulin is known to contain a greater percentage of higher-length chains, which have a wider range of application than the medium-length chain inulin found in JA tubers [7]. However, the medium-chain length inulin in JA makes it more suitable for hydrolytic and fermentative processes compared to that of chicory inulin.

Jerusalem artichoke has good tolerance to frost and drought, resistant to pest and diseases and can thrive in most soils with limited fertilizer application [8]. These features relieve its geographical limitations as it is known to show competitive growth rates even in soils where most food crops cannot grow [9]. Furthermore, the JA tuber produces higher inulin yield (0.36-12.6 ton/ha dry weight) than chicory (5.6-7.8 ton/ha dry weight) [10]. The resilience and

the impressive inulin yield of the JA tuber make it of special interest for commercial IOS production to meet the growing demands.

In a report, the production cost per hectare of the Jerusalem artichoke was estimated as 4800 €/ha, 20% more than the cultivation of potatoes, which accounts for the extra cost of expensive seeds, extra row cleaning and harvest and transport of the aerial biomass. The other cultivation processes are said to be similar to that of potato production [10]. An economic survey was conducted on the production of Jerusalem artichoke tuber in Canada. The highest cost of production (including transportation and storage) of JA tubers was Canadian $ 3800/ha [6]. In Australia, the annual variable cost of producing JA tubers purposely for ethanol production was estimated as Australian dollars $1606/ha (Australian $ 880/ha) [6].

The JA tuber also contains about 15 - 16% (dry weight) proteins [7], [11]. These proteins find application as nutraceuticals or animal feed [12], [13]. It is projected that the economic feasibility of IOS production from the JA tuber may be improved if other products can be generated alongside the main product (IOS) in a biorefinery concept. The tuber residues after IOS production contains residual carbohydrates which can be converted to biofuels using simple conversion technologies [8]. In a preliminary economic analysis conducted, revenue generation was maximized when protein, succinic acid and biogas were produced from JA in a biorefinery concept [10].

To the best of the authors' knowledge, a comprehensive techno-economic evaluation of IOS production from JA tubers is not present in open literature. The closest there is to such an evaluation, is that of the short-chain fructooligosaccharides (scFOS) counterpart production from sucrose. The present study seeks to fill that gap by conducting a vivid economic analysis on the utilization of JA tubers in a biorefinery concept, specifically for the production if IOS together with possible co-products that may enhance the economic viability of the IOS production. In this study various biorefinery configurations of IOS, protein, ethanol and biogas production from JA tubers are simulated in Aspen Plus® simulation software and economically investigated to ascertain the economic feasibility of the various biorefinery scenarios. The robustness of the JA biorefinery scenarios was tested by varying some key economic parameters in a sensitivity analysis.

7.2 Methodology

7.2.1 Simulation development methodology

Aspen Plus® V8.8 (Aspen Technology Inc., USA) software has been used in simulating a wide spectrum of biorefineries in literature and was also deployed in modeling the biorefinery scenarios evaluated in this study [14]–[16]. The NRTL property method was determined as the appropriate property method in predicting the thermo-physical properties of components. It is important to note that some essential components required for the simulation models are yet to be included in the Aspen database therefore the property database compiled by the National Renewable Energy Laboratory (NREL, USA) was used in specifying the user-defined components (Table S1 of Appendix D).

7.2.2 Economic methodology

Economic analysis provides the avenue to evaluate the real-life feasibility of simulated processes in order to make informed executive decisions [17]. The equipment costs of units such as heat exchangers, pumps, flash drums and compressors were estimated by the Aspen Plus Economic Analyzer®. The remaining units were estimated from vendor quotes and literature by updating the required sizing and cost year (2016), using the sizing index and the Chemical Engineering Plant Cost Index (CEPCI) where required. The total equipment purchase cost (TEPC) provided the seed from which the fixed capital investment (FCI) and Total capital investment (TCI) were estimated. The capital investments estimated have an accuracy range of ± 30% which is acceptable for these preliminary evaluations of the investigated scenarios [18], [19]. The FCI was determined by the summation of the of the total direct cost (TDC) and the total indirect cost (TIC), while the TCI was calculated by adding the FCI, the working capital (WC), and the cost of land. The WC was assumed as 5% of the FCI and the cost of land was estimated as 8% of the TEPC [19]. These estimations were carried out using the costing sheet developed by Choi and Lee, [20] with minor modifications.

The total operating cost (TOC) was estimated as the summation of the variable operating cost (VOC) and the fixed operating cost (FOC). The variable operating cost consists of the cost of utilities, raw materials and waste management. The costs of utilities were obtained from Aspen Plus by specifying the utility type required to meet the various unit operations. The

costs of raw materials were obtained from suppliers' quotes and prices from literature which were updated using the relevant indexes [14], [21]. The fixed operating cost is dependent on the design capacity of the biorefinery and consists of the operating labor cost, labor overhead, maintenance cost, property taxes and insurance. The labor overhead was estimated as 90% of the total operating labor costs. The maintenance cost was estimated as 3% of the biorefinery installed equipment cost. The property taxes and insurance were estimated as 0.7% of the FCI [22].

A real-term discounted cash flow rate of return (DCFROR) analysis was performed on the various biorefinery scenarios using the FCI and TOC. Table 7.1 provides a summary of the economic assumptions applied in this study. Due to the degree of uncertainty associated with the market selling price of the IOS which is the main product of interest, the minimum IOS selling price (MSP) approach was used as the measure of profitability. The MSP was estimated by iterating the IOS selling price until a net present value (NPV) of zero was obtained at an internal rate of return (IRR) of 9.7%. The effect of varying certain key parameters on the MSP was also investigated by conducting a sensitivity analysis.

Table 7.1: Economic assumptions

Description	Value and reference
Plant financing equity	100% [15]
Plant life	25 years [23]
Annual operating hours	7920 hours
Acceptable minimum %IRR (real term)	9.7% [23]
Income tax	28% [23]
Annual depreciation rate	4% [23]
Plant salvage value	Zero [23]
Start-up time	Zero years [24]
Costing year	2016
Exchange rates	US$1 = 14.51 ZAR, €1 = 15.46 ZAR
Selling price of protein	1.25 $/kg [25]
Market price of IOS	5 $/kg [14]
Selling price of residues for animal feed	0.85 $/kg [26]
Selling price of biogas	0.42 $/Nm^3 [27]
Selling price of ethanol	0.51 $/kg [10]
Cost of enzyme	5.90 $/kg of protein [25]
Cost of JA tubers	0.024 $/kg [6]
Liquids treatment/disposal	5.9 $/ton [28]
Solids treatment/disposal	33.5 $/ton [28]

7.2.3 Process overview

Biorefinery capacity is an important parameter which plays a critical role in the economic viability of the biorefineries. Based on the price and potential production rate, IOS is the major contributor to the generated revenue of the biorefinery. Therefore, the JA tuber biorefinery was sized using the production scale of IOS with careful consideration of the present market of about 200000 tonnes per annum [29]. The prebiotic market is still relatively small compared to sugar and other bioproducts. However, it shows tremendous potential for growth with reported annual growth rate of 15% [30]. In view of this, an IOS production target

of 2000 tonnes per annum (tpa) was set, which is enough to augment the current market in meeting the growing global demands for IOS without resulting in oversupply [4], [14].

The composition and type of JA tuber feedstock can have significant effects on the process design and economics as it may influence the design of components in the biorefinery process [31]. JA tuber may vary in composition due to the variety, region, harvest time, conditions and time of storage [7]. The dry weight composition of JA tuber used in this study was 65.8% inulin, 4.4% monomer sugars, 15.0% protein, 8.6% cellulose and 6.2% ash and other components, which were obtained from experimental compositional analysis of JA tubers obtained from Glen Agricultural College, Free State, South Africa in Chapter 6. This composition collectively constituted 20% (w/w) wet weight of the fresh tubers. These values are a good representation of the ranges reported in literature [7], [8], [10], [11].

To comprehensively investigate the economic viability of the different JA tuber biorefinery options, five scenarios were developed as follows:

- **Scenario A**: Extraction of protein followed by IOS production and residues sold as animal feed (Base case).
- **Scenario B**: Direct enzymatic hydrolysis of the JA tuber to produce IOS and residues sold as animal feed.
- **Scenario C**: Separate extraction of protein and inulin (for IOS production) and residues to be sold as animal feed.
- **Scenario D**: Scenario A with biogas production from the residues instead of being sold as animal feed.
- **Scenario E**: Scenario A with ethanol production from the residues instead of being sold as animal feed.

Scenario A can be considered as the base case in the biorefinery application of the JA tuber. Scenarios B and C have been included to ascertain the effects of the variations in the protein and IOS production process on the biorefinery economics. Scenarios D and E provide insights on the effect of bioenergy production from the residues on the economics of the biorefinery.

7.2.3.1 Scenario A: Extraction of protein followed by IOS production and residues sold as animal feed

The fresh JA tubers are delivered to a crusher at a rate of 2857 kg/h where the tubers are crushed into a slurry. The slurry (steam S9 in Figure 7.1) is transferred into a tank (MIXER in Figure 7.1), where it is further diluted with 952 kg/h of water to meet solids loading of 15% (w/v) [13]. The pH of the slurry is also adjusted to 5.0 while maintaining the temperature at 25 ˚C. Under these conditions, selective dissolution of proteins is optimized, whereas maintaining the structural and functional integrity of the proteins [13]. The slurry containing the dissolved protein is passed through a pressure filter (SEPARATE in Figure 7.1) where the liquid fraction (stream S7 in Figure 7.1) containing the dissolved proteins is separated from the solid residue (stream S6 in Figure 7.1). It is worth mentioning that about 30% of the inulin in the tubers is coextracted with the protein, under the conditions for optimal extraction of proteins as was seen in Chapter 6. The liquid fraction from the pressure filter is sent to a membrane separator (PROSEP in Figure 7.1) where the protein is purified by removal of the contaminant inulin.

The solid fraction from the pressure filter is sent to a tank (MIXER2 in Figure 7.1) together with the inulin separated from the protein in the membrane separator. The content of the tank is diluted with 3029 kg/h of water to obtain 5% (w/v) inulin concentration [32]. The content of the tank is preheated to 60 ºC while being fed to the IOS production reactor (REACTOR in Figure 7.1) [1]. An endoinulinase enzyme is added to the reactor content and the reaction is allowed to proceed for 4 h under constant stirring. The IOS reactor was modelled as a stirred tank reactor with operation conditions extrapolated from experimental work on optimization of IOS yields from JA tubers in Chapter 6. The reaction is terminated by quickly and briefly heating the reaction mixture to 90 ºC [33]. The reactor products consisting of IOS, glucose, fructose, unconverted inulin, cellulose and fibers is transferred to a blending tank (TANK in Figure 7.1) and allowed to cool down.

The effluents from the tank are centrifuged to separate the soluble sugars from the recalcitrant carbohydrates and fibers. The soluble sugars (stream S15 in Figure 7.1) containing IOS, glucose, fructose and unconverted inulin are sent to the simulated moving bed chromatography (SMB) column where the mono- and disaccharides are separated from the

polysaccharides. The eluent water (stream WATER3 in Figure 7.1) enters the SMB at 2832 kg/h for desorption of the sugars from the column. The SMB is made up of four columns connected in series and charged with the cation exchange resin Amberlite™ 1320 Ca, which is well adapted for separation of polysaccharides from the mono- and disaccharides [34]. The extract (stream S20 in Figure 7.1) containing the IOS in solution is dried into powder by the spray dryer. The spray dryer is modeled in Aspen using a combination of unit operation blocks (DRYER, HEATER and COMP in Figure 7.1). The product is dried by spraying into a long hollow column along with hot air (stream S52 in Figure 7.1) at 180 ^{o}C [35]. The raffinate from the SMB, which is made up of mono- and disaccharides, is mixed with the solid fraction from the centrifuge and dried in an evaporator at 104 ^{o}C. The evaporator is modeled using a combination of unit operation blocks (EX3 and EVAPOT in Figure 7.1) to achieve the phase separation and energy requirements of the process. The dried product finds economic value by being sold as animal feed.

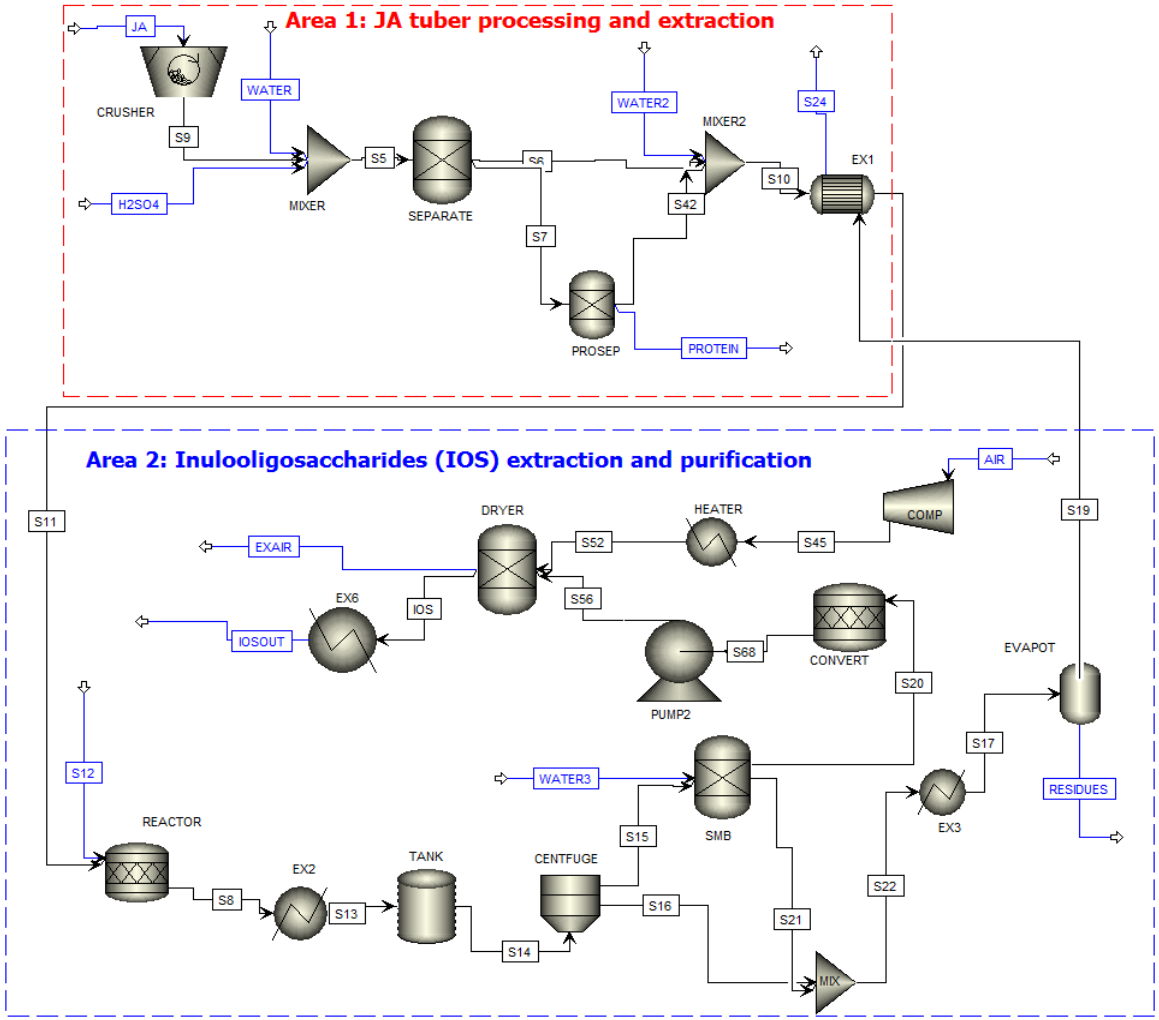

Figure 7.1: Process flow diagram of extraction of protein followed by IOS production and residues sold as animal feed (scenario A)

7.2.3.2 Scenario B: Direct enzymatic hydrolysis of the JA tuber to produce IOS and residues sold as animal feed

The configuration of unit operations in the inulooligosaccharides production and purification area in this scenario is identical to that of scenario A except for differences in equipment capacities. Figure 7.2 displays the process flow diagram of scenario B. In this scenario, 2707 kg/h of fresh JA tubers are crushed into a slurry and further diluted with 4325 kg/h of water (stream WATER in Figure 7.2) in a blending tank (MIXER in Figure 7.2) to a substrate concentration of 5% (w/v) of inulin content. The mixture in the blending tank is preheated to 60 ^{o}C and fed in to the IOS reactor (Reactor in Figure 7.2) without any prior protein extraction. The protein content of the JA tuber constitutes part of the residues sold as animal feed.

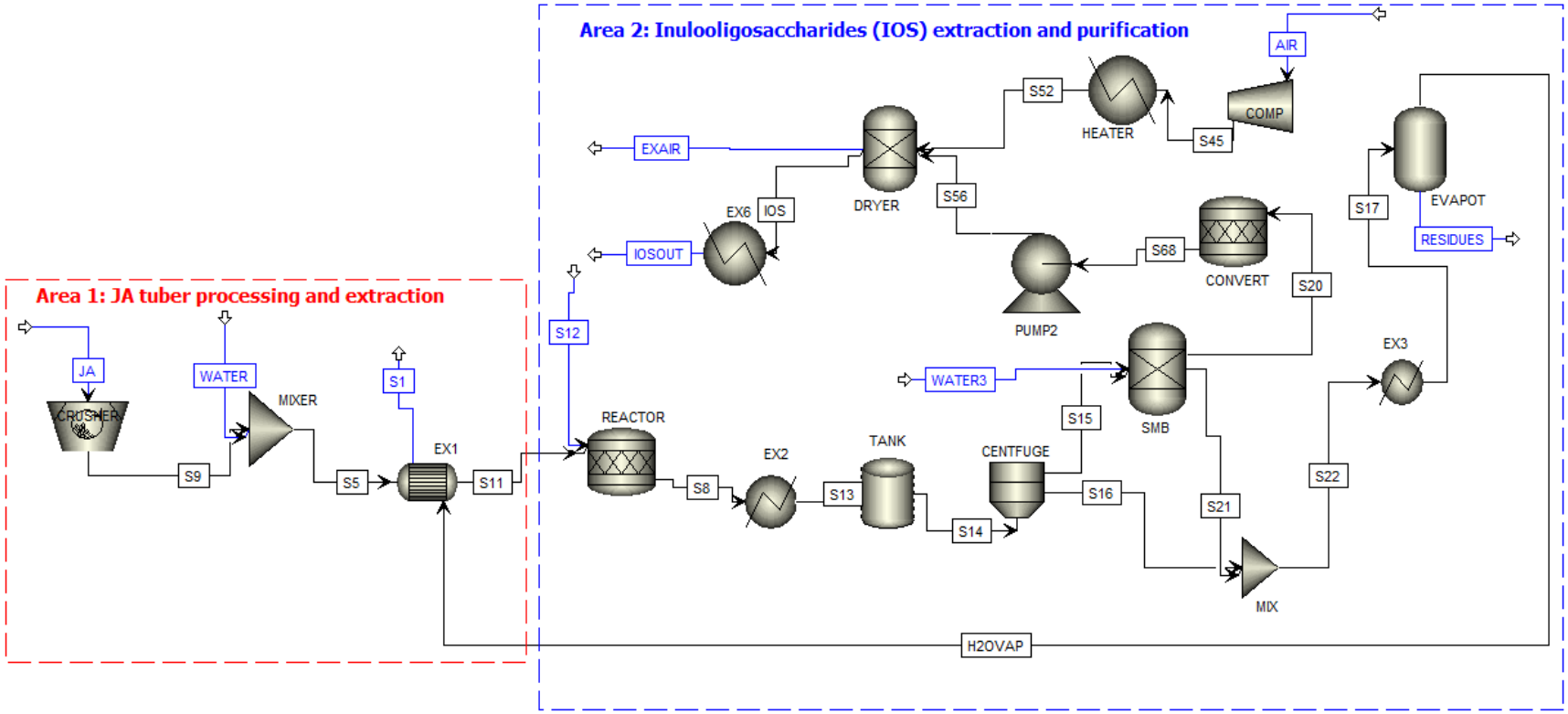

Figure 7.2: Process flow diagram of direct enzymatic hydrolysis of the JA tuber to produce IOS and residues sold as animal feed (scenario B)

7.2.3.3 Scenario C: Separate extraction of protein and inulin (for IOS production) and residues to be sold as animal feed

The configuration of unit operations in the inulooligosaccharides production and purification area is identical to that of scenario A, with changes in equipment capacities. In this scenario, 2960 kg/h of fresh JA tubers are crushed into a slurry and transferred into a blending tank (MIXER in Figure 7.3). The content of the tank is diluted to a solids loading of 15% (w/v) with the pH adjusted to 5.0 by addition of H_2SO_4 while maintaining the temperature at 25 ^oC. The content of the tank is fed to a pressure filter to extract the liquid fraction (mainly proteins with some coextracted inulin) from the solid residue. The extracted liquid is fed to a membrane separator to separate the proteins from the coextracted inulin.

The solid residues from the pressure filter are fed to a blending tank (MIX6 in Figure 7.3) with the solids loading adjusted to 10% (w/v) with 2821 kg/h of water (stream S37 in Figure 7.3). The pH of the slurry is adjusted to 7.0 by addition of NaOH. The slurry is preheated to 70 ^oC using a heat exchanger (EX4 in Figure 7.3). These conditions are appropriate for optimal extraction of inulin [13]. The slurry is passed through a pressure filter (INULSEP in Figure 7.3) to separate the liquid fraction (mainly inulin) from the solid residues (mainly cellulose and fibers). The extracted inulin fractions (streams S40 and S42 in Figure 7.3) are mixed in a blending tank and the substrate concentration adjusted to 5% (w/v) of inulin, while being preheated to 60 ^oC before being fed to the IOS reactor. The solid residues (stream S41 in Figure 7.3) from the inulin extraction process are mixed with the mono- and disaccharides stream (stream S21 in Figure 7.3) from the SMB and sent to the evaporator for drying.

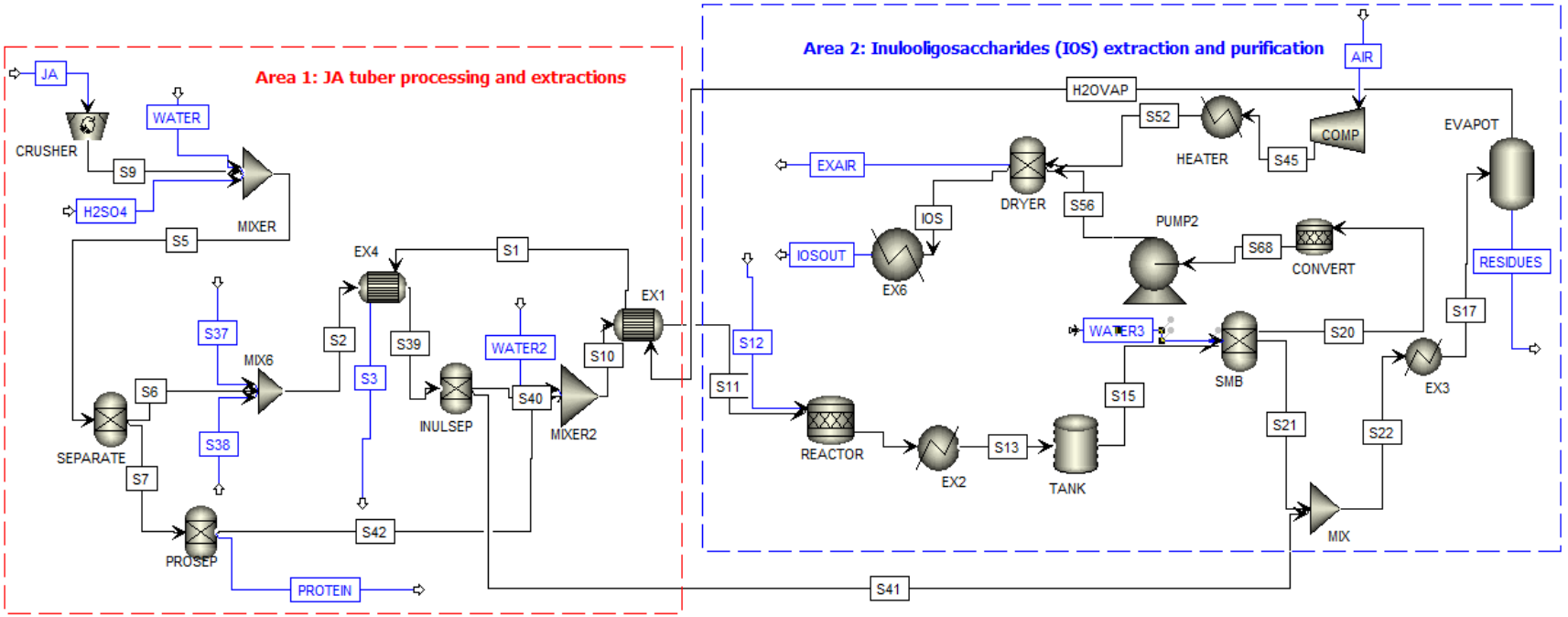

Figure 7.3: Process flow diagram of separate extraction of protein and inulin (for IOS production) and residues to be sold as animal feed (scenario C)

7.2.3.4 Scenario D: Scenarios A with biogas production from the residues instead of being sold as animal feed

In this scenario, the raffinate from the SMB (SMB in Figure 7.4) and the solid fraction from the centrifuge (CENTFUGE in Figure 7.4) are blended in a tank (MIX in Figure 7.4) and cooled down to 32 ˚C by a heat exchanger (EX5 in Figure 7.4). The cooled stream is fed directly to the anaerobic digester (BIODIGE2 and BIODIGES in Figure 7.4). In the anaerobic digestion, 91% of each organic component is broken down and 86% converted to biogas (stream BOIGAS in Figure 7.4), which is essentially methane and carbon dioxide [31]. These two biogas components are produced at almost equimolar amounts [31]. The biogas produced finds application as a fuel for heat and electricity generation or can be upgraded to biomethane. The liquid from the biodigester (stream WWT in Figure 7.4) containing soluble organics is disposed for wastewater treatment.

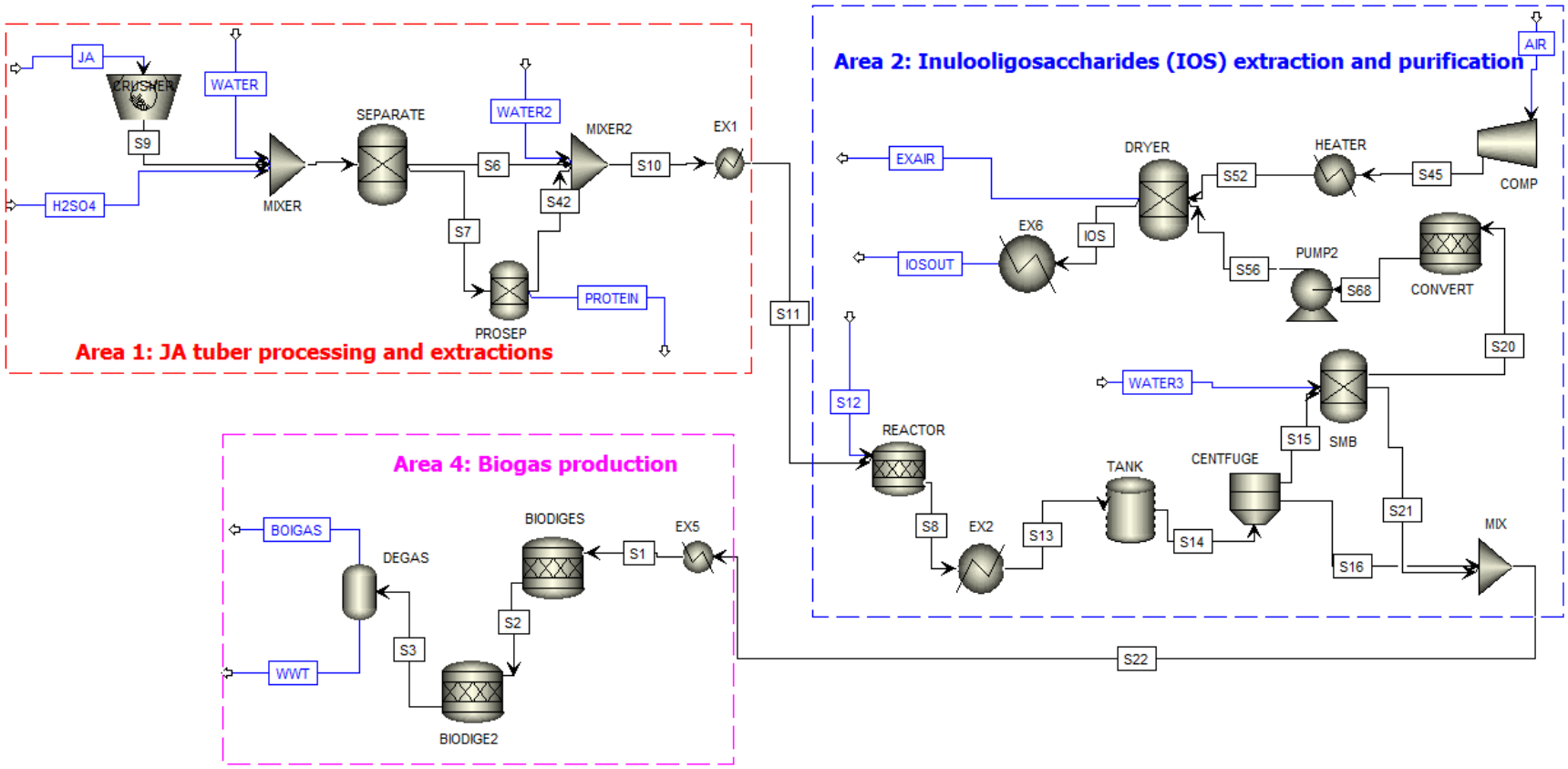

Figure 7.4: Process flow diagram of extraction of protein followed by IOS production and biogas production from residues (scenario D)

7.2.3.5 Scenario E: Scenarios A with ethanol production from the residues instead of being sold as animal feed

This scenario has the protein extraction and inulooligosaccharides production and purification sections identical to scenario A except for variations in equipment capacities. The raffinate from the SMB (SMB in Figure 7.5), which is made up of the mono- and disaccharides, is mixed with the solid fraction from the centrifuge (CENTFUGE in Figure 7.5). The resulting stream is concentrated in an evaporator (EVAPOT in Figure 7.5) before being fed to the SSF reactor (HYDLSS and FERMENT in Figure 7.5) for ethanol production. In the SSF reactor, cellulose and inulin are broken down to monomer sugars (glucose and fructose) using a cocktail of exoinulinase and cellulase enzymes. This process is referred to as enzymatic saccharification or hydrolysis. The cellulase enzymes preparation breaks down the cellulose fibers into cellobiose and finally into glucose monomers. The exoinulinase enzyme essentially breaks off fructose from the non-reducing ends of inulin resulting in glucose and fructose monomers. Simultaneously the monomer sugars produced are fermented to ethanol by S. *cerevisiae.* The hydrolysis and fermentation reactors are modeled as separately in Aspen Plus (HYDLSS and FERMENT in Figure 7.5) in order to appropriately account for the energy requirement of the unit operations. However, in real life the two processes occur simultaneously in a single reactor at 35 ^{o}C for 96 h [36]. Two main effluents exit the SSF reactor. The gaseous effluent (stream S3 in Figure 7.5) mainly composed of carbon dioxide and nitrogen. The other stream (stream S2 in Figure 7.5) is the fermentation broth containing ethanol, water, unconverted sugars, dissolved gases, yeast and metabolites. A flash drum (FEMDEG in Figure 7.5) was included in the Aspen Plus simulation to achieve the phase separation of the gases from the fermentation broth. However, it is not required in real life and therefore not included in the equipment cost.

The fermentation broth from the SSF reactor is sent to the beer column (BEERCOL in Figure 7.5), where the dissolved CO_2 is removed as the overhead stream (Stream S1 in Figure 7.5). The ethanol and water are removed as the side stream (stream L411 in Figure 7.5) and bottom stream (stream SOLIDWASTE in Figure 7.5) contains the solid residues. The side stream from the beer column is sent to the rectification column (RECTC in Figure 7.5), where the ethanol is

concentrated to a near-azeotropic composition. The ethanol stream (stream NS12 in Figure 7.5) from the rectification column is then sent to an adsorption column (MOLSIEVE in Figure 7.5) charged with molecular sieves. Here the ethanol is further dehydrated to 99.5% [37]. The beer column overhead and the gaseous streams from the SSF reactor (mainly CO_2 and some ethanol) are both fed to a water scrubber (SCRUBBER in Figure 7.5), where the trapped ethanol is recovered and recycled to the beer column.

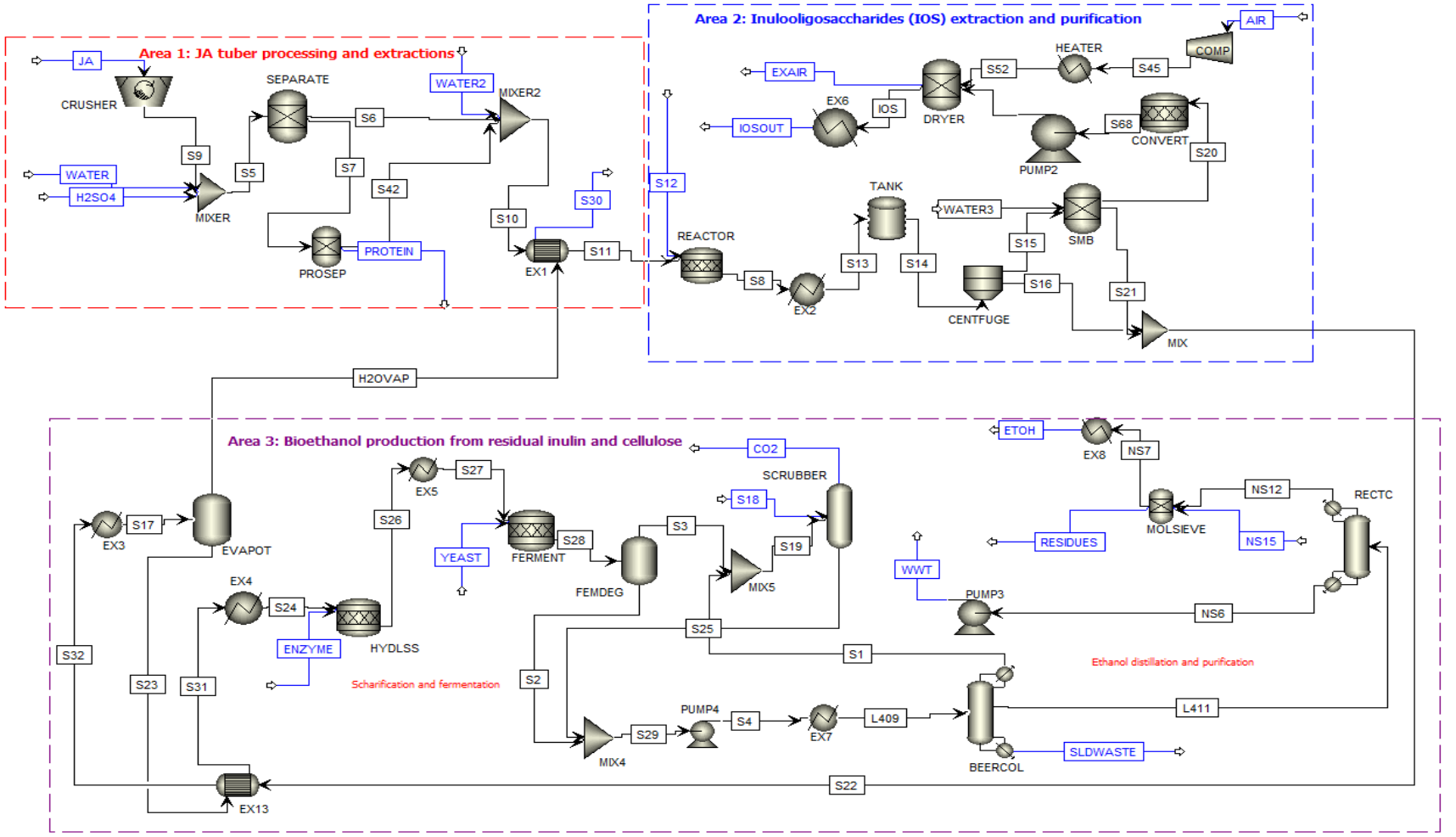

Figure 7.5: Process flow diagram of extraction of protein followed by IOS production and ethanol production from residues (scenario E)

7.3 Results and discussion

7.3.1 Mass and energy balances

Table 7.2 provides a summary of the mass and energy balances for the investigated scenarios. IOS is the key product of the developed scenarios with production target of 2000 tpa. The estimated yields of IOS from scenarios A, B, C, D and E were 76.3, 76.3, 77.1, 76.3 and 76.3 w/w_{inulin} respectively, which are based on experimental data of IOS yields from JA tubers in Chapter 6. To meet the IOS production demand of 2000 tpa, supplies of 22627, 21439, 23443, 22627 and 22627 tpa of fresh JA tubers were required by scenarios A, B, C, D and E respectively. Scenario B required the least amount of fresh JA tubers because it had the least inulin losses, due to the absence of any extraction or separation process prior to feeding JA tubers to the IOS reactor. Consequently, scenario B produced the greatest amount (2515 tpa) of residues with intended usage as animal feed.

Scenario C required the greatest amount of JA tubers to meet the IOS production target despite having the highest yield of IOS on inulin. It was noticed that the additional inulin losses (5%) due to prior extraction of the inulin before hydrolysis in the IOS reactor, outweighed the increased yield (1%) from the absence of interaction by the fibers. As a result of the increased JA feedstock, the amount of protein extracted in scenario C was 4% more than that produced in scenarios A, D and E. To valorize the residues in cases where the market may not be available for sale as animal feed, scenarios D and E were introduced to explore the prospects of producing biogas and bioethanol from the residues respectively. In scenario D, 1316456 normal cubic meter (Nm^3) of biogas per year is produced, which translates into about 24770 GWh electricity equivalent per year; this is significantly less than the capacity of a typical biogas plant for processing agricultural waste [38]. In scenario E, about 0.23 million US gallons of ethanol per year is produced, a quantity which is about 1% of typical commercial scale bioethanol production plant [31], [39], [40]. Tables S2 – S7 of the Appendix D provides a detailed balance of components for the various biorefinery scenarios.

Table 7.2: Summary of mass and energy balance for 2000 t per annum IOS production by JA refinery scenarios

Item	Unit	Scenario				
		A	B	C	D	E
Raw materials						
JA tubers	tpa	22627	21439	23443	22627	22627
Enzyme	tpa	206	195	213	206	317
Process water	tpa	46118	55472	76761	46118	46316
Others	tpa	8	-	16	8	119
Products						
IOS	tpa	2000	2000	2000	2000	2000
Protein	tpa	625	-	648	625	625
Animal feed	tpa	2486	2515	2258	-	-
Biogas	Nm^3/yr	-	-	-	1316456	-
Ethanol	tpa	-	-	-	-	673
Utilities						
Electricity demand	kW	1752	1657	1815	1752	1752
Heating demand	kW	4963	5323	6296	1865	5229
Cooling demand	kW	536	551	604	937	941

Expectedly, scenario C required the most electricity of 14378 MWh, mainly due to the increased plant capacity in order to accommodate the increased feedstock rate required to meet the set IOS production targets. The reverse was seen in the case of scenario B, which required the least electricity of 13127MWh per year as a result of requiring the least amount of feedstock to meet the set IOS production target. In all scenarios, over 95% of the total electricity demand was associated with the spray dryer. Because a very large amount of compressed air was required to dry the IOS product into powder form as it was fed into the drying chamber through the atomizer [35]. A similar trend was also noticed for the production of short-chain fructooligosaccharides (scFOS) from sucrose [14], [34]. The electricity demands reported here are about 10 times greater

compared to that reported for equivalent scales of scFOS production from sucrose (Chapter 5). The most likely reason being the increased equipment capacity as a result of low the inulin substrate concentration of 5% (w/v) used for IOS production compared to the 60% (w/v) used for sucrose substrate. The relative solubility of sucrose is significantly higher than that of inulin [3]. The spray dryer and the evaporator were the two most heat intensive equipment in all studied scenarios collectively accounting for over 90% of the total heating demands. The spray dryer required high pressure steam (HP steam) for heating, while the evaporator required low pressure steam (LP steam) as the heating medium. Scenario C recorded the highest heating demand of 6296 kW due to the increased capacity, while scenario D resulted the least heating demand of 1865 kW due to the absence of an evaporator in its process. Figure 7.6 provides the hourly utility usage data for the studied scenarios. Scenario E required the highest amount of HP steam (3.61 t/h), because of additional heating required by the reboilers of the ethanol distillation columns, while scenario C required the highest amount of LP steam (7.55 t/h) due to increased tonnage of residues to be dried in the evaporator.

Scenario E had the highest cooling demand of 941 kW followed by scenarios D, C, B and A with 937, 604, 551 and 536 kW respectively. Scenarios D and E have exceptionally high cooling demands due to chilled water, which was needed to cool the biodigester and SSF reactor respectively. Also over 60% of the cooling water demand in all scenarios was associated with the IOS reactor. The inulin hydrolysis reaction seems to be an exothermic reaction therefore, requiring cooling water to maintain the reaction temperature at 60 ^{o}C. Scenario D required the highest chilled water (20.49 t/h), which was solely required for cooling of the biodigester in order to maintain the temperature at 32 ^{o}C. Considering the significant amount of heat energy (262.3 kW) generated by the anaerobic digestion process, some authors have suggested the possibility of utilizing the heat energy for other applications using an absorption chiller [41].

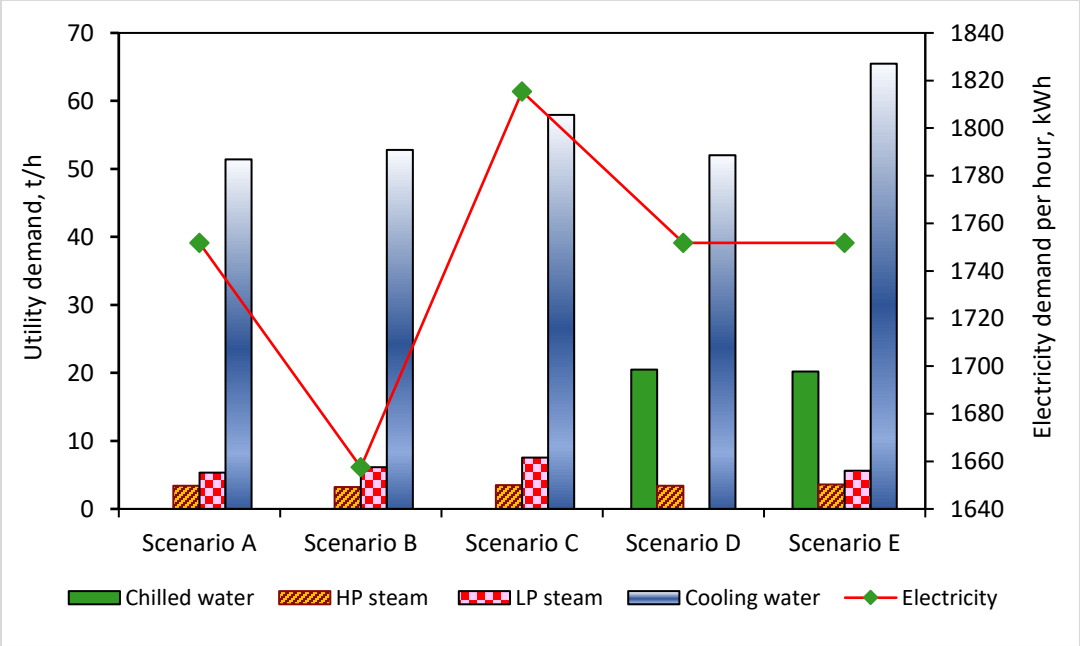

Figure 7.6: Hourly utility usage for all studied scenarios to meet the 2000 tpa IOS production target

7.3.2 Economic evaluation

7.3.2.1 Capital estimation

The mass and energy balances provided the basis for equipment sizing and estimation of the total equipment purchase cost (TEPC). The total capital investments (TCI) were then estimated from the TEPC using a costing worksheet [20]. Table 7.3 provides a summary of the capital investment estimation for the various scenarios. The IOS production and purification section is the major contributor to the TEPC for all case scenarios. The biogas and the bioethanol production sections contributed 22% and 29% to the TEPCs of scenarios D and E respectively. Due to the elimination of the protein and inulin extraction steps in scenario B, there was no need for the associated equipment hence the significant reduction (20%) in the TEPC compared to the base case (scenario A).

The TEPCs of scenarios D and E were 32% and 45% higher than that of the scenario A due to the inclusion of the biogas and bioethanol production sections for processing of the residues. The TEPC of scenario C was 12% more than that of scenario A as a result of the increased feedstock demand to meet the set IOS production target. The increased feedstock demand to meet the set IOS production target translated into additional capital investment due to the corresponding increase in equipment capacity to meet the processing demand.

Table 7.3: Summary of capital estimation for the studied scenarios

Item	**Scenario**				
	A	**B**	**C**	**D**	**E**
Equipment cost	*Cost (million US$)*				
JA tuber processing and extractions	1.82	0.04	2.86	1.82	1.82
IOS production and purification	5.59	5.43	5.16	5.55	5.54
Bioethanol production	-	-	-	-	3.03
Biogas production	-	-	-	2.04	-
Total equipment purchase cost (TEPC)	*7.41*	*5.47*	*8.02*	*9.41*	*10.38*
Installation (70% of TEPC)	5.18	3.83	5.61	6.58	7.27
Process piping (35% of TEPC)	2.59	1.91	2.81	3.29	3.63
Instrumentation (40% of TEPC)	2.96	2.19	3.21	3.76	4.15
Insulation (3% of TEPC)	0.22	0.16	0.24	0.28	0.31
Electrical (10% of TEPC)	0.74	0.55	0.80	0.94	1.04
Buildings (45% of TEPC)	3.33	2.46	3.61	4.23	4.67
Yard improvement (15% of TEPC)	1.11	0.82	1.20	1.41	1.56
Auxiliary Facilities (40% of TEPC)	2.96	2.19	3.21	3.76	4.15
Total plant direct cost (TPDC)	*26.52*	*19.58*	*28.72*	*33.67*	*37.17*
Engineering (25% of TPDC)	6.63	4.89	7.18	8.42	9.29
Construction (35% of TPDC)	9.28	6.85	10.05	11.78	13.01
Total plant indirect cost (TPIC)	*15.91*	*11.75*	*17.23*	*20.20*	*22.30*
Contractor's fee (5% of [TPDC + TPIC])	2.12	1.57	2.30	2.69	2.97
Contingency (10% of [TPDC + TPIC])	4.24	3.13	4.59	5.39	5.95
Other costs (OTC)	*6.36*	*4.70*	*6.89*	*8.08*	*8.92*
Fixed capital investment (FCI)	*48.79*	*36.02*	*52.84*	*61.95*	*68.40*
Working capital (WC)	*2.44*	*1.80*	*2.64*	*3.10*	*3.42*
Total capital investment (TCI)	*51.23*	*37.82*	*55.48*	*65.05*	*71.82*

Figure 7.7 displays the contribution of the cost of certain major equipment to the TEPC. The spray dryer accounted for a significant percentage of the TEPC in all five biorefinery scenarios. Similarly, the spray dryer accounted for over 65% of the TEPC of scFOS production plants with identical production capacities (Chapter 5). Usually, most heat and electricity intensive equipment such as spray dryer, boiler and steam treatment are among the most expensive equipment in a process plant [14], [42]. The cost of reactor vessels accounted for 25% and 28% of the TEPC of scenarios D and E respectively, because of the additional reactors required for the anaerobic digestion and ethanol productions respectively. The pressure filters accounted for 34% of the TEPC of scenario C as a result of the additional filter required for the inulin extraction prior to hydrolysis in the IOS reactor. The trends in the TCI, FCI and WC are similar to that of the TEPC, since it constituted the seed from which that other investments were determined. The capital investments obtained here are about four times higher than that obtained for an equivalent scale of scFOS production from sucrose (Chapter 5). This is partly due to the inclusion of extra equipment for the production of the accompanying products and also the increased equipment sizes as a of result the low inulin substrate concentration.

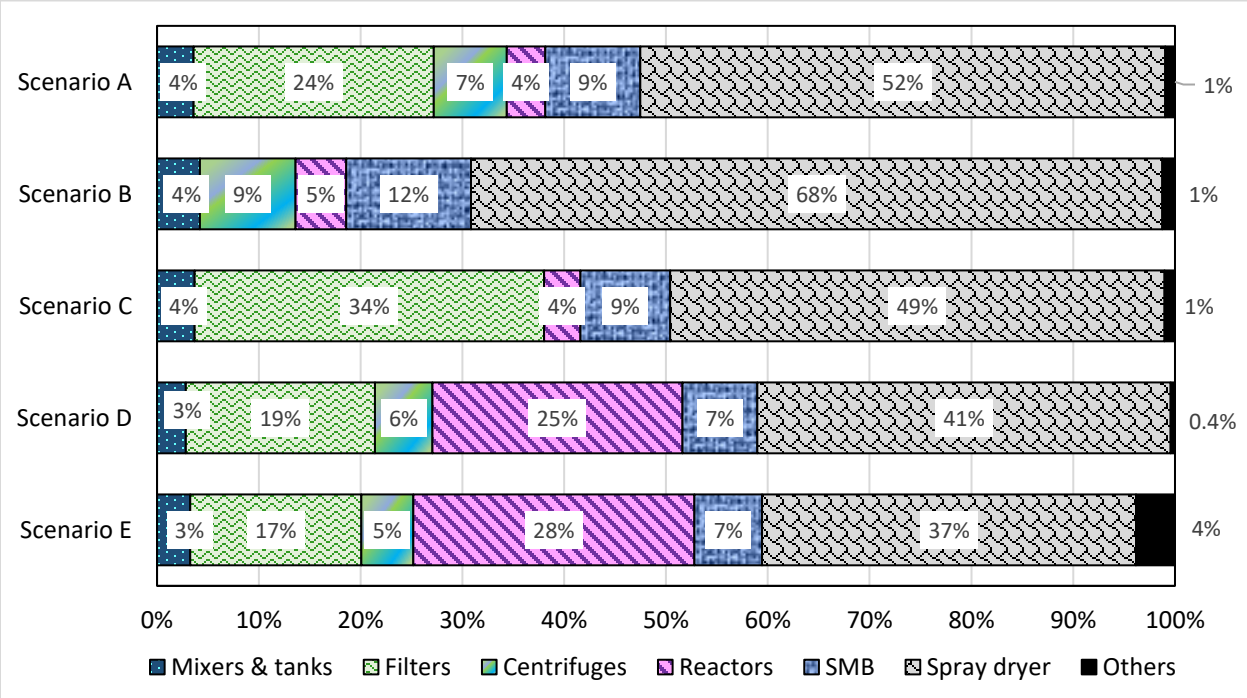

Figure 7.7: Percentage contribution of individual equipment cots to the total equipment cost

7.3.2.2 Estimation of operating cost

Figure 7.8 provides a summary of the total operating cost (TOC) estimation. The mass and energy balances and plant sizing constituted the basis for the TOC estimation. In all case scenarios, the cost of enzyme alone accounted for over 70% of the raw material cost which also accounted for over 47% of the variable operating cost (VOC) (Figure 7.9A). In other reports, enzyme cost accounted for about 20% - 46% of the total operation cost of producing bioethanol from lignocellulosic materials [25], [31]. It was indicated that the cost of enzyme can be somewhat reduced by on site enzyme production rather than purchase of enzyme from suppliers [25]. The additional enzyme requirement for hydrolysis in the SSF reactor resulted in scenario E recording the highest cost of enzyme (1.87 M$/yr) and consequently the highest raw material cost of 2.44 M$/yr. The second highest contributor to the VOC was the utility cost of which the highest contributor (above 60%) was electricity in all case scenarios. Scenarios D, E and C recorded high VOCs due to the increased utility costs associated with the additional residue processing steps in scenarios D and E and the increased feedstock tonnage in scenario C. The trend in the fixed operating cost (FOC) followed that of the fixed capital investment as maintenance cost, insurance and taxes were estimated as fractions of the FCI and ISBL. Scenario E therefore had the highest FOC of 2.63 M$/yr by virtue of having the highest FCI. The TOC values obtained for the JA biorefinery scenarios are almost twice those obtained for equivalent scales of scFOS production from sucrose in Chapter 5. The main components responsible for the increase were the high utility costs as well as the components of the FOC that were dependent on the capital investment.

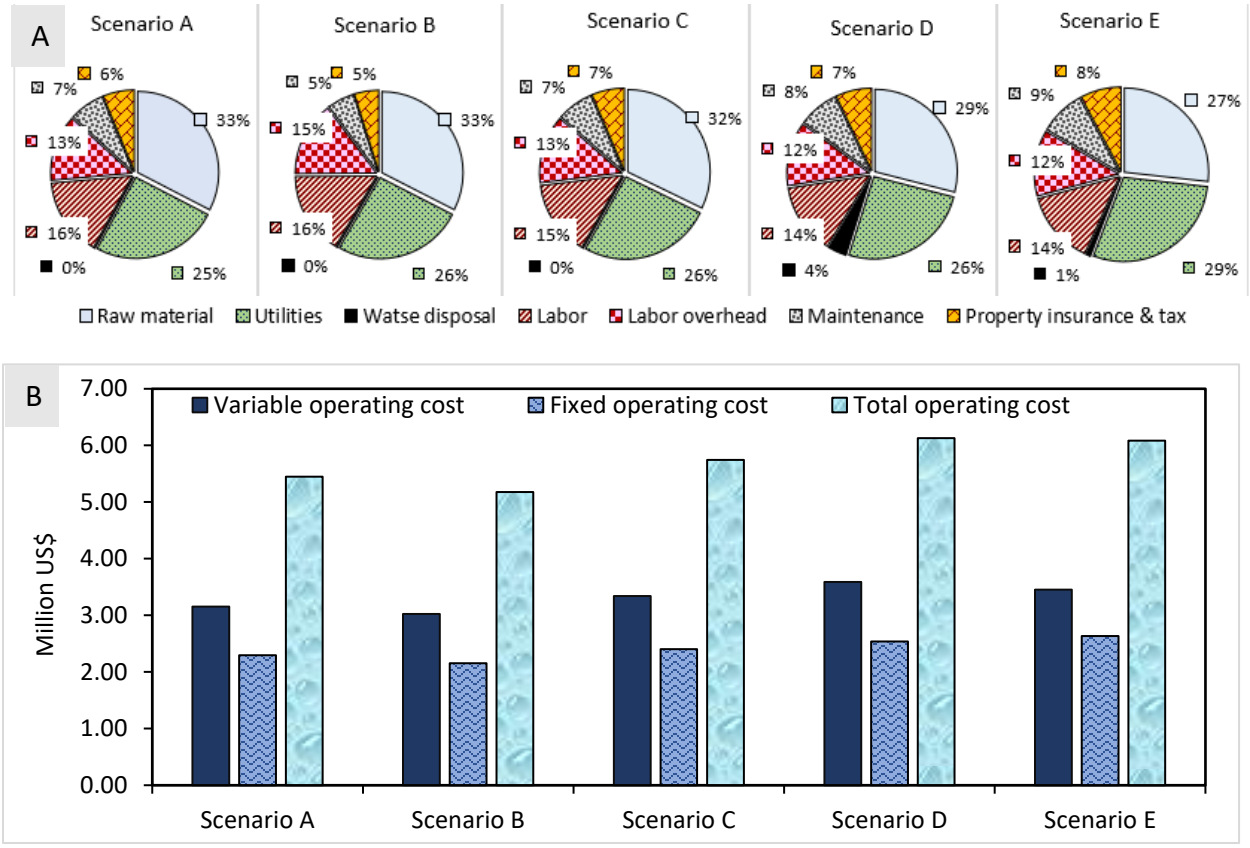

Figure 7.8: Summary of total operating cost. A - Percentage contribution of the various components of the total operating cost. B – Estimations of the variable, fixed and total operating costs.

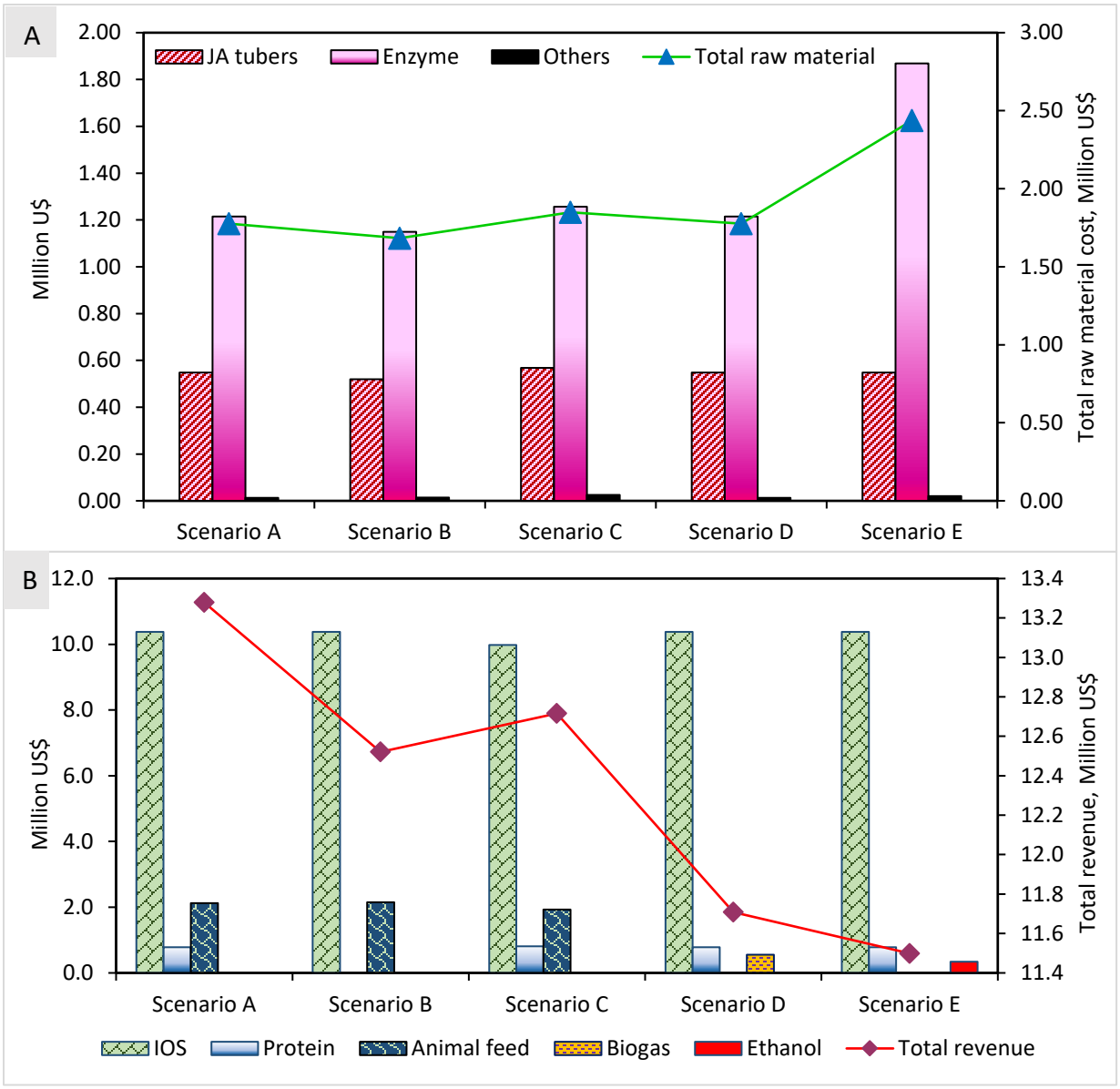

Figure 7.9: A-Estimation of raw material cost, B-contribution of the various products to the total revenue

7.3.3 Profitability analysis

The results of the profitability analysis shed light on the economic performance as one of the key drivers for sustainability of the biorefineries. The MSP of all scenarios were estimated and compared to the set IOS market price of 5 $/kg (Figure 7.10). Of all the scenarios studied, Scenario B was the most profitable as it recorded the least MSP of 3.91 $/kg. Scenario A also showed a slight margin of profitability with an MSP of 4.57 $/kg. Scenarios C, D and E cannot be considered

as profitable since their MSPs of 5.25, 6.53 and 7.44 $/kg respectively, were higher than the set market price. Scenarios E especially was the least attractive because the additional capital and operating expenditure associated with the ethanol production section far outweighs the revenue (Figure 9B) generated from ethanol sales and consequently resulting in a deficit on the profitability of the entire process. Evidently, this scale of ethanol production is not profitable especially when the ethanol is to be sold at the competitive market price [23]. It is therefore important to properly consider the rationale of coproduct selection as this has significant implications on the biorefinery economics. Some coproducts cannot generate a high enough revenue to offset the additional capital and operating costs associated with the additional processing steps [43].

Scenario D also was not profitable as a result of the additional capital and operating investments of the biogas production section while the revenue generated from the biogas was only 5% of the total revenue (Figure 7.9B) thereby resulting in an economic deficit. Even though scenario D does not demonstrate profitability for commercialization, it provides potential for a self-energy sufficient biorefinery as the biogas generated may be used to meet some of the heating and electricity demands [17], [44].

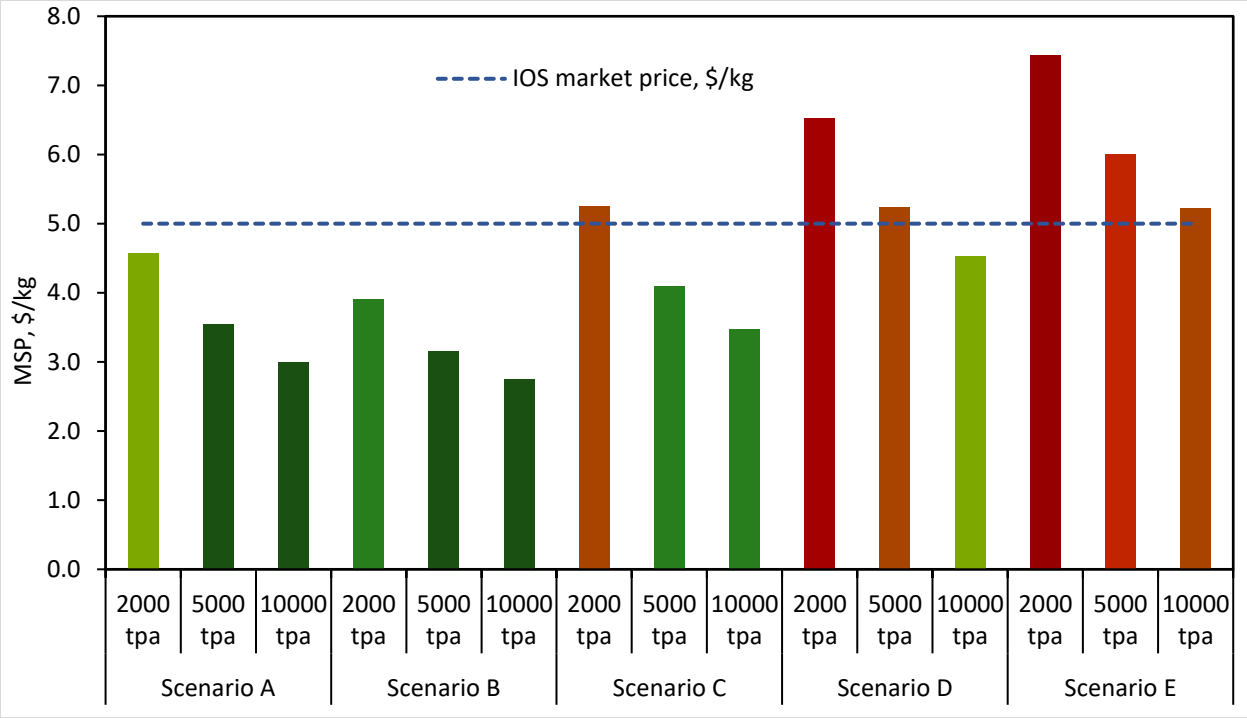

Figure 7.10: Profitability (MSP) for the various biorefinery scenarios at 2000, 5000 and 10000 tpa IOS production targets

Scenario B had the advantage of requiring the least capital and operating investment (20% less than scenario A) making it the most profitable scenario. It also gives an indication of the fact that a plant dedicated to sole production of IOS from JA tubers still has a good margin of profitability. The MSP obtained for scenario B is greater (above 25%) than the MSPs obtained for equivalent scales of powdered scFOS production from sucrose (Chapter 5) indicating the inferior profitability of the former. This in mainly because the JA tuber biorefinery in scenario B required about twice the capital investment of the sucrose-scFOS systems for equivalent production targets. Scenario A showed less profitability compared to scenario B because the expenditure associated with the protein extraction and purification step outweighed the revenue generated from the protein sales. Scenario C generated similar amounts of revenue as scenario A however, the additional capital and operating costs from the increased tonnage of feedstock due to the addition of the inulin separation step led to the non-profitability. Therefore, best IOS production method under the investigated conditions is by the direct hydrolysis of the JA tuber. This prevents the increased

feedstock tonnage in compensation for the inulin losses which occur from protein and inulin pre-extractions.

The effect of IOS production scale on MSP was investigated by also estimating the MSP of all scenarios at IOS production targets of 5000 and 10000 tpa (Figure 7.10). Compared to the 2000 tpa production target, the MSPs of all scenarios experienced at least 19% and 30% reductions at 5000 and 10000 tpa production scales respectively. As a result, scenario C achieved some margin of profitability from 5000 tpa and higher. Scenario D also demonstrated probability only at 10000 tpa. Scenario E on the other hand was not profitable even at 10000 tpa. The ranking of profitability between scenarios was the same for all the IOS production scales investigated.

7.3.4 Sensitivity analysis

Sensitivity analysis was carried out to ascertain the impact of certain key economic parameters on the MSP of the various scenarios investigated. The change in profitability was measured for 20% variations in the economic parameters. The results are displayed in Figure 7.11. For all scenarios the MSP were highly sensitive to changes in the FCI. Scenario B maintained profitability even with a 20% increase in FCI indicating some resilience to marginal changes in the capital investment. The IRR also greatly influences the MSP, a 20% increase in IRR resulted in at least 11% increase in MSP for all scenarios.

The selling prices of ethanol and biogas had minimal effects on the scenarios E and D as they contribute very little to the net revenue. Interestingly all scenarios were less sensitive to the cost of raw materials (JA tubers and enzyme costs). This resilience of the biorefinery process to changes in raw material cost provides potential for long term financial sustainability of the plant [39]. Therefore, the raw material costs would not introduce much uncertainty in the profitability of the biorefinery process [45].

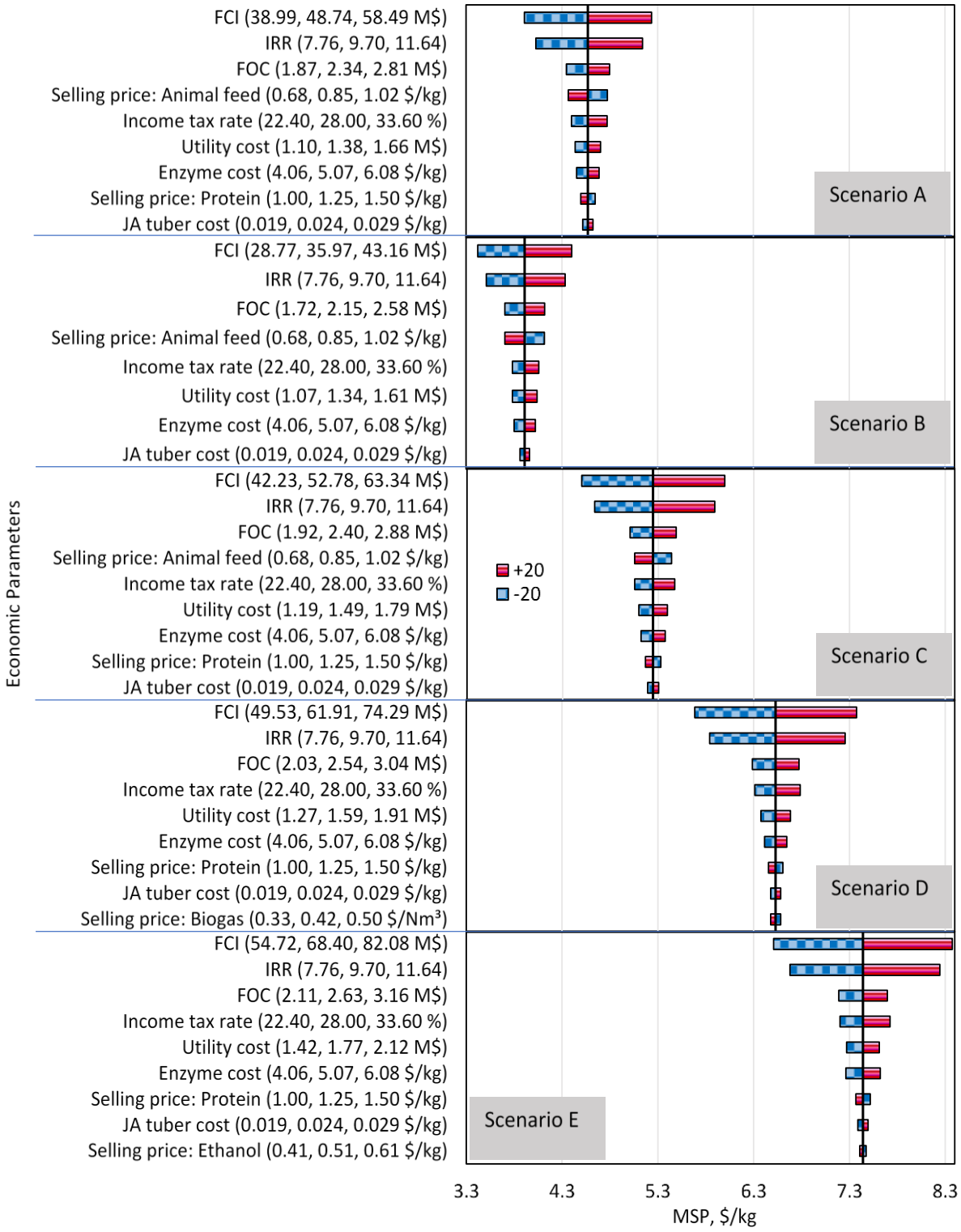

Figure 7.11: Sensitivity analysis for biorefinery case scenarios

7.4 Comparison of best cases of the sucrose and JA tuber biorefineries

The scFOS and IOS products closely resemble each other in terms of physicochemical properties as well as in functional and prebiotic properties and therefore may be used as alternatives to each other [4]. The techno-economic analysis of the scFOS and IOS production process scenarios provides the opportunity to make a well-informed decision with regards to which product has the technical and economic advantage as far as meeting the growing demand for these prebiotics is concerned. Part of objective 6 was to compare the economics of the best-case scenario of scFOS production from sucrose with the best case of the IOS production from the JA tuber biorefinery in the biorefinery concept. Table 7.4 presents the summary of the economic analyses for the sucrose and JA tuber biorefinery scenarios to be compared. As was seen in Chapter 5, the most economical sucrose biorefinery was the free enzyme (FE) system, where the β-fructofuranosidase enzyme was applied in soluble form for the production of scFOS from sucrose and the by-product sugars sold as animal feed. The best case of the JA tuber biorefineries was the scenario B where IOS was produced and the residue sold as Animal feed.

The FE-system of scFOS production, demonstrated superior economic feasibility compared to that of the scenario B from the JA tuber biorefineries as the estimated MSP of the FE-system is far smaller than that of the scenario B (Table 7.4). This is also corroborated by the NPV and IRR values of the FE-system which were almost twice that of the scenario B. One of the main factors responsible for this is the difference in substrate concentration of the feedstocks during oligosaccharides production (Table 7.5). The sucrose substrate concentration was 60% (w/v) whereas that of the inulin was 5% (w/v). The inulin substrate concentration was low due to the viscosity constraints from the presence of the cellulosic fibres in the JA tuber. Also, inulin has a lower solubility compared to sucrose, which places a limitation on how much inulin can be solubilized in aqueous media for IOS production. The low solubility translated into higher equipment sizes hence the high FCI of the JA biorefinery scenarios compared to that of the sucrose-scFOS systems. The low substrate concentration also influenced the utility consumption and cost, as energy demand is proportional to the amount of water to be removed from very

dilute process streams. Especially in the case of the spray dryer where much more electricity and heating were required to produce the IOS in powdered form.

Interestingly, the raw material cost of sucrose (1.4 Million US$) required to meet the production target was less than that of the JA tubers (0.54 Million US$), mainly because the per kilogram cost of sucrose is greater than that of the JA tuber. The β-fructofuranosidase for scFOS production is usually limited by two factors: (I) the transfructosylating activity of the enzyme is only expressed in the presence of a high enough concentration of sucrose above 10% (w/v) [46]. (II) the accumulation of glucose in the reaction medium during scFOS formation results in inhibition of the enzyme thereby limiting the yield of scFOS obtained [47]. Because of this about 7% (w/v) of unconverted sucrose is usually present at the end of the reaction [48]. The endoinulinase enzyme on the other hand is not limited by by-product inhibition hence the higher product yield obtained with the JA tuber inulin compared to that of sucrose. However, increasing the inulin substrate concentration above 5% (w/v) usually results in a decrease in the IOS yield mainly because of the solubility constraints [49]. The reaction time for maximum IOS yield in this study was also significantly shorter than that for scFOS from sucrose (4h vs 6h).

Even though the FE system of the scFOS production from sucrose shows more economic feasibility compared to the scenario A of the JA tuber biorefinery, the JA tuber biorefinery may offer some socio-economic benefits especially in the agricultural sector. The cultivation of JA tubers would be encouraged since it is not capital intensive like many food crops, and this would create a source of livelihood for the farmers. Moreover, the aerial biomass of the JA plant also holds some value which may enhance the economic feasibility of the biorefinery.

Table 7.4: Summary of economic analysis for 2000 tpa of scFOS and IOS production from sucrose and JA tubers respectively

Biorefinery scenarios			**TCI**	**TOC**	**NPV**	**IRR**	**MSP**
	Name	**Description**	Million US $	Million US $	Million US $	%	$/kg
Sucrose	FE-system	scFOS and animal feed using free enzyme system	15.45	3.40	31.73	32.83	2.61
	CAIE-system	scFOS and animal feed using calcium alginate immobilized enzyme system	15.95	3.50	30.65	31.42	2.69
	AIE-system	scFOS and animal feed using Amberlite IRA 900 immobilized enzyme system	16.19	3.97	27.34	28.89	2.94
JA tuber	Scenario A	IOS, protein and animal feed	51.23	5.45	5.95	11.16	4.57
	Scenario B	IOS and animal feed	37.82	5.18	15.11	14.57	3.91
	Scenario C	IOS (from inulin extract), protein and animal feed	55.48	5.74	-3.29	8.93	5.25
	Scenario D	IOS, protein and biogas	65.05	6.13	-21.24	5.21	6.53
	Scenario E	IOS, protein and ethanol	71.82	6.08	-33.89	2.91	7.44

Table 7.5: Summary of reaction conditions for scFOS and IOS best-case scenarios

	Sucrose	**JA tuber**
Parameter	Free enzyme (FE) system	IOS, protein and animal feed biorefinery (scenario B)
Reaction time (h)	6	4
Feedstock demand	3280 tpa of sucrose	22627 tpa of fresh JA tuber
Substrate concentration	60% (w/v) sucrose	5% (w/v) inulin
Product yield	61 %($w/w_{sucrose}$)	77.1% (w/w_{inulin})

7.5 Conclusion

The production of IOS was investigated with possible economic improvement by co-product generation. Scenarios A and C investigated the various biorefinery configurations of IOS, protein and animal feed production from the tubers. Scenario B investigated the sole production of IOS from the JA tuber with sale of the residues as animal feed. Scenarios D and E explored the economics of biogas and ethanol productions respectively from the residues designated for animal feed. Of all scenarios investigated, scenario B was the most profitable with an MSP of 3.91 $/kg as it required the least capital investment for equivalent revenue generation. Results from scenarios A and C suggest that the coproduction of protein rather introduces an economic deficit on the IOS production process. Also, as was seen from scenarios D and E, further processing of the residues for biofuel production is not advisable at the 2000 tpa production scale. Interestingly all scenarios were least sensitive to the cost of raw materials (JA tubers). This resilience of the biorefinery process to changes in raw material cost provides potential for long term financial sustainability. When compared to the best case of the counterpart scFOS production from sucrose, the best case of IOS production from JA tuber (scenario B) demonstrated inferior profitability mainly due to inulin solubility limitations associated with the latter.

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Chapter 8

8 Conclusions and recommendations

8.1 Overview of chapters with novel contributions and key findings

The study was to investigate, optimize and compare the economic feasibilities of sucrose and Jerusalem artichoke tubers as feedstocks for scFOS and IOS production respectively. The immobilized and free enzymes systems were investigated in the pursuit of a more economical system of scFOS production form sucrose. Multiproduct biorefinery scenarios were also investigated in order to develop a more economical process of IOS production from JA tubers. The introduction in Chapter 1 and the literature review in Chapter 2 provided the overview of enzyme immobilization, scFOS production from sucrose and Jerusalem artichoke tuber as a multiproduct biorefinery feedstock.

To date, the novel enzyme used in this study had not been immobilized, neither has the Amberlite IRA 900 ion exchange resin been used as an immobilization material in scFOS production from sucrose. Furthermore, the techno-economic comparison of scFOS production using the free and immobilized enzyme systems is yet to be carried out. Also, the optimization of IOS production from the various inulin fractions obtainable from JA tubers with protein extraction consideration in a biorefinery concept has not been explored before. In addition, no detailed techno-economic evaluation of multiproduct biorefinery to enhance the economic viability of IOS production from JA tubers has been found in open literature. The following objectives were therefore developed:

- Objective 1: Immobilization of β-fructofuranosidase using three suitable support materials namely: Amberlite IRA 900 and Dowex marathon MSA anion exchange resins and calcium alginate beads followed by characterization of the immobilized enzymes from the three supports, in terms of enzyme activity recovery and enzyme immobilization efficiency.

» Objective 2: Comparison of the performance (product yields) of the immobilized enzyme to the free enzyme in the production of scFOS of composition identical to Actilight®.

» Objective 3: Assessing the re-usability of the immobilized enzyme and the regeneration capacity of the support materials used for the immobilization procedure.

» Objective 4: Comparative techno-economics study of scFOS production from sucrose using the free and immobilized enzyme systems.

» Objective 5: Optimization of the conversion of inulin in the inulin-rich substrates resulting from the alternative scenarios of inulin and protein co-extraction from JA tubers, into IOS a high value marketable product, through the application of endoinulinase enzyme.

» Objective 6: Economic evaluation of various biorefinery scenarios applicable to the conversion of Jerusalem artichoke tubers, and comparison of the effective scFOS production costs in such scenarios to the best cases for scFOS production from sucrose.

In Chapter 4, *Amberlite IRA 900 versus calcium alginate in immobilization of a novel, engineered β-fructofuranosidase for short-chain fructooligosaccharide synthesis from sucrose,* the novel β-fructofuranosidase was immobilized on Amberlite IRA 900 and Dowex Marathon MSA ion exchange resins and calcium alginate beads. The immobilization results from Amberlite IRA 900 and calcium alginate beads were sufficient, and the immobilized enzymes were further tested against the free enzyme counterpart in scFOS production from sucrose. The reusabilities of the immobilized enzymes were also tested.

The **novel contributions** in this chapter includes the successful immobilization of a novel β-fructofuranosidase and the application of Amberlite IRA 900 immobilized enzyme in scFOS production from sucrose. The **key finding** was that the calcium alginate immobilized enzyme demonstrated superior scFOS yield, while the Amberlite IRA 900 immobilized enzyme

demonstrated better reusability and consistency of product yield and composition. This demonstrated some potential for savings on the cost of the high value enzyme by the reusability of the immobilized enzymes.

In Chapter 5, *Comparison of immobilized and free enzyme systems in industrial production of short-chain fructooligosaccharides from sucrose using techno-economic approach*, the immobilization data in Chapter 4 was extrapolated to develop economic models to assess the most industrially viable option (immobilized or free enzyme) at a production target of 2000 tonnes of scFOS per annum from sucrose. All three enzyme scenarios demonstrated economic feasibility with the free enzyme system being the most profitable with the least MSP of 2.61 $/kg of powdered scFOS. The **novel contribution** in this chapter include the design, simulation and techno-economic evaluation of the various systems of scFOS production from sucrose. Also, the comparison between the free enzyme and immobilized enzyme systems were carried out.

The **key finding** from this chapter is that even though the immobilized enzymes demonstrated good reusability as seen in Chapter 4, which presupposes some potential economic advantage due to the savings on the cost of enzyme as a result of immobilization, this benefit was not enough to offset the additional costs associated with immobilizing the enzyme, especially due to the indirect cost of a reduced scFOS yield as a result of immobilization. Also, because the enzyme production section constituted a small component of the overall cost of the scFOS production process.

In Chapter 6, *Optimization of inulooligosaccharides production from inulin-rich substrates extracted from Jerusalem artichoke (Helianthus tuberosus L.) tubers,* IOS production from Jerusalem artichoke tubers was considered as an alternative to scFOS production from sucrose. Various inulin-rich substrates were obtained from the JA tuber with consideration to protein extraction and subjected to enzymatic hydrolysis using endoinulinase. The reaction conditions were optimized for each substrate to obtain the highest IOS yields on inulin. The results obtained with the JA inulin-rich substrates were benchmarked with that from pure chicory inulin.

The **novel contributions** in this Chapter includes the preparation of the various inulin-rich samples obtainable from JA tubers with consideration to protein extraction in a biorefinery scenario. Also, optimization of the IOS production was carried out on the various substrates independently to maximize the IOS yields. The **key finding** is that significant IOS yields were obtained from the JA inulin-rich substrates with the JA powder producing the highest overall IOS yield of 52.6% (w/$w_{dry\ JA\ powder}$). The JA tuber demonstrated sufficiency as an alternative inulin source for IOS production by producing yields comparable to that from pure chicory inulin.

In Chapter 7, *Techno-economic analysis of inulooligosaccharides, protein and bioenergy co-production from Jerusalem artichoke tubers in a biorefinery concept,* the data from experimental work in Chapter 6 was implemented in investigating the most economically viable multiproduct biorefinery configuration for production of 2000 tonnes per annum of IOS from JA tubers. Of all the scenarios investigated, only scenarios A and B demonstrated some margin of profitability. The **novel contribution** in this chapter includes the design, simulation and techno-economic analysis of the multiproduct biorefinery for producing IOS, protein, animal feed and biofuel using JA tuber as feedstock. Also, the comparison between sucrose and JA tubers as feedstocks for production of the short-chain fructose-containing oligosaccharides.

The **key findings** from this chapter is that the dedicated production of IOS from the JA tuber with the sale of the residues was the most profitable of the JA multiproduct biorefineries. The coproduction of protein and bioenergy production from the residues introduced an economic deficit as the associated expenditure outweighed the revenue generated. The comparison of the best-case scenarios of the sucrose and JA tuber biorefineries revealed the free enzyme system of scFOS production from sucrose as the more profitable venture.

8.2 Recommendations

» Future research could be directed towards exploring the performance of the Amberlite IRA 900 and calcium alginate immobilized enzymes in a continuous process, preferably a packed column reactor. This may enhance the reusability hence improving the economic feasibility of the immobilized enzyme systems.

» The economic modelling in this study affirms the possibility of a viable scFOS production in South Africa using the immobilized or free enzyme systems. Considerations should therefore be given to the establishment of an scFOS production unit in South Africa in order to generate additional revenue from the sugar industry and create employment.

» Further research should be conducted on improving the solubility of the JA tuber inulin as that would help increase the substrate concentration of the feedstock for IOS production. This would improve the economic feasibility by leading to reduction in capital investment and operating costs due to the reduction in equipment sizes to meet the set production targets. Consideration should also be given to the utilization of the entire JA plant in the biorefinery to obtain an energy self-sustaining biorefinery.

» The economic evaluations conducted on the JA tuber biorefineries provides sufficient indication that establishing a JA biorefinery in South Africa would assist in meeting the world's growing demand for IOS. This venture would provide employment especially in the agricultural sector through the cultivation of JA.

Appendix A: Supplementary information

Amberlite IRA 900 versus calcium alginate in immobilization of a novel, engineered β-fructofuranosidase for short-chain fructooligosaccharide synthesis from sucrose

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Table S1: ANOVA analysis. Effect estimates of the factors affecting the percentage protein adsorption by ion exchange resins

Factor	Effect Estimates; Var.:%Adsorption; R-sqr=.26927; Adj:0. (Design: 2**(3-0) design (Spreadsheet1) in Workbook1) 2**(3-0) design; MS Residual=158.1019 DV: %Adsorption									
	Effect	Std.Err.	t(16)	p	-95.% Cnf.Limt	+95.% Cnf.Limt	Coeff.	Std.Err. Coeff.	-95.% Cnf.Limt	+95.% Cnf.Limt
Mean/Interc.	50,1667	2,566628	19,54575	0,000000	44,7257	55,60768	50,16667	2,566628	44,7257	55,60768
(1)Temp	-10,7933	5,133256	-2,10263	0,049168	-21,6754	0,08868	-5,39667	2,566628	-10,8377	0,04434
(2)Type	2,6133	5,133256	0,50910	0,617632	-8,2687	13,49535	1,30667	2,566628	-4,1343	6,74768
(3)Treatment	0,7850	5,133256	0,15292	0,880370	-10,0970	11,66702	0,39250	2,566628	-5,0485	5,83351
1 by 2	-0,0700	5,133256	-0,01364	0,989289	-10,9520	10,81202	-0,03500	2,566628	-5,4760	5,40601
1 by 3	-1,7083	5,133256	-0,33280	0,743608	-12,5904	9,17368	-0,85417	2,566628	-6,2952	4,58684
2 by 3	-0,8983	5,133256	-0,17500	0,863273	-11,7804	9,98368	-0,44917	2,566628	-5,8902	4,99184
1*2*3	-5,2617	5,133256	-1,02502	0,320604	-16,1437	5,62035	-2,63083	2,566628	-8,0718	2,81018

Appendix B: Supplementary information

Comparison of immobilized and free enzyme systems in industrial production of short-chain fructooligosaccharides from sucrose using techno-economic approach

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Table S1: Properties of the user-defined components used in the scFOS production process

Component	Property	Quantity	Units	Comments
Protein	Formula	$CH_{1.57}O_{0.31}N_{0.29}S_{0.007}$		
	MW	22.8396	g/mol	
	DHSFRM	-17618	cal/mol	
Enzyme	Formula	$CH_{1.59}O_{0.42}N_{0.24}S0.01$		Properties were used to represent the β-fructofuranosidase enzyme.
	MW	24.0156	g/mol	
	DHSFRM	-17618	cal/mol	
GF2	Formula	$C_{18}H_{32}O_{16}$		The only available property data were the formula and MW. Their molecular structures were drawn in Aspen Plus which was used to estimate their thermodynamic properties.
	MW	504.438	g/mol	
GF3	Formula	$C_{24}H_{42}O_{21}$		
	MW	666.579	g/mol	
GF4	Formula	$C_{30}H_{52}O_{26}$		
	MW	828.727	g/mol	
ZYMO	Formula	$CH_{1.8}O_{0.5}N_{0.2}$		*Z. mobilis* has the average composition of several microorganisms. Properties were used to represent *P. pastoris*
	MW	24.6264	g/mol	
	DHSFRM	-31169.39	cal/mol	
Sources: Adopted from Humbird, D. et al., Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol, National Renewable Energy Laboratory (NREL), Golden, CO, 2011; http://mastersearch.chemexper.com				

Table S2: Composition of a 1L volume of Pichia trace salts (PTM1) solution

Component	**Amount**
$CuSO_4.5H_2O$	6.0 g
NaI	0.08 g
$MnSO_4.H_2O$	3.0 g
$Na_2MoO_4.2H_2O$	0.2 g
H_3BO_3	0.02 g
$CoCl_2$	0.5 g
$ZnCl_2$	20.0 g
$FeSO_4.7H_2O$	65 g
Biotin	0.2 g
H_2SO_4	5 mL
Water	Add to a final volume of 1L

Table S3: Pre-inoculum and inoculum for β-fructofuranosidase production

Component	**Volume**		**Sterilization**
	Pre-inoculum	**Inoculum**	
dH_2O	2.76 ml	27.6 ml	Autoclave
1M Phosphate buffer, pH 6.0	0.4 ml	4 ml	
10x Yeast nitrogen base (YNB)	0.4 ml	4 ml	Filter sterilise
500x Biotin	8 µl	80 µl	
10% Glycerol	0.4 ml	4 ml	

Table S4: Cost of raw materials

Material	**Unit price, $/kg**
Glycerol	0.610
$CaSO_4$	0.600
K_2SO_4	0.700
KOH	1.200
NH_4OH	0.310
WATER	0.001
PICHIA	6.000
$CuSO_4.5H_2O$	1.800
NaI	200.000
$MnO_4S.H_2O$	0.640
$Na_2MoO_4.2H_2O$	100.000
H_3BO_4	0.900
$Co\text{-}Cl_2$	100.000
$ZnCl_2$	1.300
$FeSO_4.7H_20$	1.000
H_2SO_4	0.290
H_3PO_4 85%	1,000
Biotin	56.000
$MgSO_4.7H_2O$	0.300
Sucrose	0.400
$CaCl_2.6H_2O$	0.120
Sodium Alginate	2.000
NaOH	0.385
Amberlite IRA 900	2.788

Table S5: Utility price

Utility	**Unit**	**Price**
Cooling Water	$/kg	0.00127
Electricity	$/kWh	0.08
Steam	$/MJ	0.0012

Table S6: Stream table for the enzyme production section to meet 2000 tonnes per annum scFOS production demand using the free enzyme system

Stream Name	**FFASE**	**ENZS3**	**ENZS4**	**ENZS5**	**EXAIR**	**PICHIA1**	**PICHIA2**
Temp C	30	30	30	25	30	30	30
Pressure bar	1.01	0.40	1.01	1.01	1.01	1.01	1.01
Total kg/hr	2.86	16.99	21.45	1.69	17.93	0.66	0.01
Mass %							
GLYCEROL	8.27	0.00	1.10	3.01	0.00	0.0001	0.0001
CASO4	0.00	0.00	0.00	0.10	0.00	0.00	0.00
K2SO4	0.00	0.00	0.00	2.59	0.00	0.00	0.00
KOH	0.00	0.00	0.00	0.17	0.00	0.00	0.00
NH4OH	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WATER	91.03	0.00	14.20	89.16	2.49	0.001	0.0009
N2	0.11	79.00	62.73	0.00	75.05	0.00	0.00
O2	0.01	21.00	4.71	0.00	5.63	0.00	0.00
FFASE	0.21	0.00	0.03	0.00	0.00	0.00	0.00
PICHIA	0.00	0.00	3.12	0.00	0.00	99.998	99.999
CUSO4	0.00	0.00	0.00	0.002	0.00	0.00	0.00
NAI	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MNO4S	0.00	0.00	0.00	0.001	0.00	0.00	0.00
NA2MOO4	0.00	0.00	0.00	0.0001	0.00	0.00	0.00
H3BO4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CO-CL2	0.00	0.00	0.00	0.0002	0.00	0.00	0.00
ZNCL2	0.00	0.00	0.00	0.008	0.00	0.00	0.00
IRON(-01)	0.00	0.00	0.00	0.02	0.00	0.00	0.00
H2SO4	0.00	0.00	0.00	0.004	0.00	0.00	0.00
H3PO4	0.00	0.00	0.00	3.49	0.00	0.00	0.00
BIOTIN	0.00	0.00	0.00	0.0001	0.00	0.00	0.00
MGSO4	0.00	0.00	0.00	1.43	0.00	0.00	0.00
CO2	0.36	0.00	14.11	0.00	16.83	0.00	0.00
Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table S7: Stream table for the scFOS production section to meet 2000 tonnes per annum of scFOS using the free enzyme system

Stream Name	**SUCROSE**	**WATER 2**	**COMPAIR**	**SMBWATER**	**SUGARS**	**HOTAIR**	**SCFOS**	**WASTEH2O**
Temp C	25	25	25	25	25	25	25	25
Pressure bar	1.01	1.01	1.01	1.01	1.01	1	1.01	1.01
Total, kg/h	442.27	294.84	7216.80	384.78	195.41	7515.43	250.56	379.10
Mass flowrate, kg/h								
AIR	0.00	0.000	7216.80	0.00	0.00	7216.80	0.00	0.00
WATER	0.00	294.84	0.00	384.78	19.37	277.63	14.61	368.02
SUCROSE	442.27	0.00	0.00	0.00	35.92	1.89	0.10	1.89
DEXTR-01	0.00	0.00	0.00	0.00	119.74	6.30	0.33	6.30
D-FRU-01	0.00	0.00	0.00	0.00	7.98	0.42	0.02	0.42
KESTOSE	0.00	0.00	0.00	0.00	5.25	5.25	99.77	0.28
NYSTOSE	0.00	0.00	0.00	0.00	5.46	5.46	103.78	0.29
GF4	0.00	0.00	0.00	0.00	1.68	1.68	31.93	0.09
FFASE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.82
Mass %								
AIR	0.00	0.00	100.00	0.00	0.00	96.03	0.00	0.00
WATER	0.00	100.00	0.00	100.00	9.91	3.69	5.83	97.08
SUCRO-01	100.00	0.00	0.00	0.00	18.38	0.03	0.04	0.50
DEXTR-01	0.00	0.00	0.00	0.00	61.28	0.08	0.13	1.67
D-FRU-01	0.00	0.00	0.00	0.00	4.09	0.01	0.01	0.11
KESTOSE	0.00	0.00	0.00	0.00	2.69	0.07	39.83	0.07
NYSTOSE	0.00	0.00	0.00	0.00	2.80	0.07	41.42	0.08
GF4	0.00	0.00	0.00	0.00	0.86	0.02	12.74	0.02
FFASE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table S8: Stream table for enzyme production to meet 2000 tpa scFOS production demand using the calcium alginate system

Stream Name	**FFASE**	**ENZS3**	**ENZS4**	**ENZS5**	**EXAIR**	**PICHIA1**	**PICHIA2**
Temp C	30	30	30	25	30	30	30
Pressure bar	1.01	0.40	1.01	1.01	1.01	1.01	1.01
Total kg/h alg	1.90	11.32	14.30	1.13	11.95	0.44	0.009
Mass %							
GLYCEROL	8.27	0.00	1.10	3.01	0.00	0.0001	0.0001
CASO4	0.00	0.00	0.00	0.10	0.00	0.00	0.00
K2SO4	0.00	0.00	0.00	2.59	0.00	0.00	0.00
KOH	0.00	0.00	0.00	0.17	0.00	0.00	0.00
NH4OH	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WATER	91.03	0.00	14.20	89.16	2.49	0.001	0.001
N2	0.11	79.00	62.73	0.00	75.05	0.00	0.00
O2	0.01	21.00	4.71	0.00	5.63	0.00	0.00
FFASE	0.21	0.00	0.03	0.00	0.00	0.00	0.00
PICHIA	0.00	0.00	3.12	0.00	0.00	99.998	99.998
CUSO4	0.00	0.00	0.00	0.002	0.00	0.00	0.00
NAI	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MNO4S	0.00	0.00	0.00	0.001	0.00	0.00	0.00
NA2MOO4	0.00	0.00	0.00	0.0001	0.00	0.00	0.00
H3BO4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CO-CL2	0.00	0.00	0.00	0.0002	0.00	0.00	0.00
ZNCL2	0.00	0.00	0.00	0.008	0.00	0.00	0.00
IRON(-01)	0.00	0.00	0.00	0.02	0.00	0.00	0.00
H2SO4	0.00	0.00	0.00	0.004	0.00	0.00	0.00
H3PO4	0.00	0.00	0.00	3.49	0.00	0.00	0.00
BIOTIN	0.00	0.00	0.00	0.0001	0.00	0.00	0.00
MGSO4	0.00	0.00	0.00	1.43	0.00	0.00	0.00
CO2	0.36	0.00	14.11	0.00	16.83	0.00	0.00
Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table S9: Stream table for 2000 tpa scFOS production using the Calcium alginate system

Stream Name	**SUCROSE**	**WATER 2**	**COMPAIR**	**SMBH2O**	**SUGARS**	**HOTAIR**	**SCFOS**	**WASTEH2O**
Temp C	25	25	25	25	25	25	25	25
Pressure bar	1.01	1.01	1.01	1.01	1.01	1.00	1.01	1.01
mass flow kg/hr	457.26	304.84	7216.80	384.78	202.04	7515.43	250.56	379.10
AIR	0.00	0.00	7216.80	0.00	0.00	7216.80	0.00	0.00
WATER	0.00	294.84	0.00	384.78	19.37	277.63	14.61	368.02
SUCROSE	442.27	0.00	0.00	0.00	35.92	1.89	0.10	1.89
DEXTR-01	0.00	0.00	0.00	0.00	119.74	6.30	0.33	6.30
D-FRU-01	0.00	0.00	0.00	0.00	7.98	0.42	0.02	0.42
KESTOSE	0.00	0.00	0.00	0.00	5.25	5.25	99.79	0.28
NYSTOSE	0.00	0.00	0.00	0.00	5.46	5.46	103.78	0.29
GF4	0.00	0.00	0.00	0.00	1.68	1.68	31.93	0.09
FFASE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.82
Mass %								
AIR	0.00	0.00	100.00	0.00	0.00	96.03	0.00	0.00
WATER	0.00	100.00	0.00	100.00	9.91	3.69	5.83	97.08
SUCRO-01	100.00	0.00	0.00	0.00	18.38	0.03	0.04	0.50
DEXTR-01	0.00	0.00	0.00	0.00	61.28	0.08	0.13	1.66
D-FRU-01	0.00	0.00	0.00	0.00	4.09	0.01	0.01	0.11
KESTOSE	0.00	0.00	0.00	0.00	2.69	0.07	39.83	0.07
NYSTOSE	0.00	0.00	0.00	0.00	2.80	0.07	41.42	0.08
GF4	0.00	0.00	0.00	0.00	0.86	0.02	12.74	0.02
FFASE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48
Total	100.00	100.00	100.000	100.00	100.00	100.00	100.00	100.00

Table S10: Stream table for enzyme production to meet 2000 tpa scFOS production demand using the Amberlite IRA 900 system

Stream Name	**FFASE**	**ENZS3**	**ENZS4**	**ENZS5**	**EXAIR**	**PICHIA1**	**PICHIA2**
Temp C	30	30	30	25	30	30	30
Pressure bar	1.01	0.40	1.01	1.01	1.01	1.01	1.01
Total kg/h alg	0.41	2.43	3.07	0.24	2.57	0.09	0.002
Mass %							
GLYCEROL	8.27	0.00	1.10	3.01	0.00	0.0001	0.0001
CASO4	0.00	0.00	0.00	0.10	0.00	0.00	0.00
K2SO4	0.00	0.00	0.00	2.59	0.00	0.00	0.00
KOH	0.00	0.00	0.00	0.17	0.00	0.00	0.00
NH4OH	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WATER	91.03	0.00	14.20	89.16	2.49	0.001	0.001
N2	0.11	79.00	62.73	0.00	75.05	0.00	0.00
O2	0.01	21.00	4.71	0.00	5.63	0.00	0.00
FFASE	0.21	0.00	0.03	0.00	0.00	0.00	0.00
PICHIA	0.00	0.00	3.12	0.00	0.00	99.998	99.998
CUSO4	0.00	0.00	0.00	0.002	0.00	0.00	0.00
NAI	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MNO4S	0.00	0.00	0.00	0.001	0.00	0.00	0.00
NA2MOO4	0.00	0.00	0.00	0.0001	0.00	0.00	0.00
H3BO4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CO-CL2	0.00	0.00	0.00	0.0002	0.00	0.00	0.00
ZNCL2	0.00	0.00	0.00	0.008	0.00	0.00	0.00
IRON(-01)	0.00	0.00	0.00	0.02	0.00	0.00	0.00
H2SO4	0.00	0.00	0.00	0.004	0.00	0.00	0.00
H3PO4	0.00	0.00	0.00	3.49	0.00	0.00	0.00
BIOTIN	0.00	0.00	0.00	0.0001	0.00	0.00	0.00
MGSO4	0.00	0.00	0.00	1.43	0.00	0.00	0.00
CO2	0.36	0.00	14.11	0.00	16.83	0.00	0.00
Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table S11: Stream table for 2000 tpa scFOS production using the Amberlite IRA 900 system

Stream Name	**SUCROSE**	**WATER2**	**COMPAIR**	**SMBH2O**	**SUGARS**	**HOTAIR**	**SCFOS**	**WASTEH20**
Temp C	25	25	25	25	25	25	25	25
Pressure bar	1.0	1.0	1.0	1.0	1.01	1.01	1.01	1.01
Mass flow kg/hr	529.00	352.70	8631.90	460.20	233.70	8989.00	299.70	453.40
Mass %								
AIR	0.00	0.00	100.00	0.00	0.00	96.03	0.00	0.00
WATER	0.00	100.00	0.00	100.00	9.91	3.69	5.83	97.08
SUCRO-01	100.00	0.00	0.00	0.00	18.38	0.03	0.04	0.50
DEXTR-01	0.00	0.00	0.00	0.00	61.28	0.08	0.13	1.66
D-FRU-01	0.00	0.00	0.00	0.00	4.09	0.01	0.01	0.11
KESTOSE	0.00	0.00	0.00	0.00	2.69	0.07	39.83	0.07
NYSTOSE	0.00	0.00	0.00	0.00	2.80	0.07	41.42	0.08
GF4	0.00	0.00	0.00	0.00	0.86	0.02	12.74	0.02
FFASE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

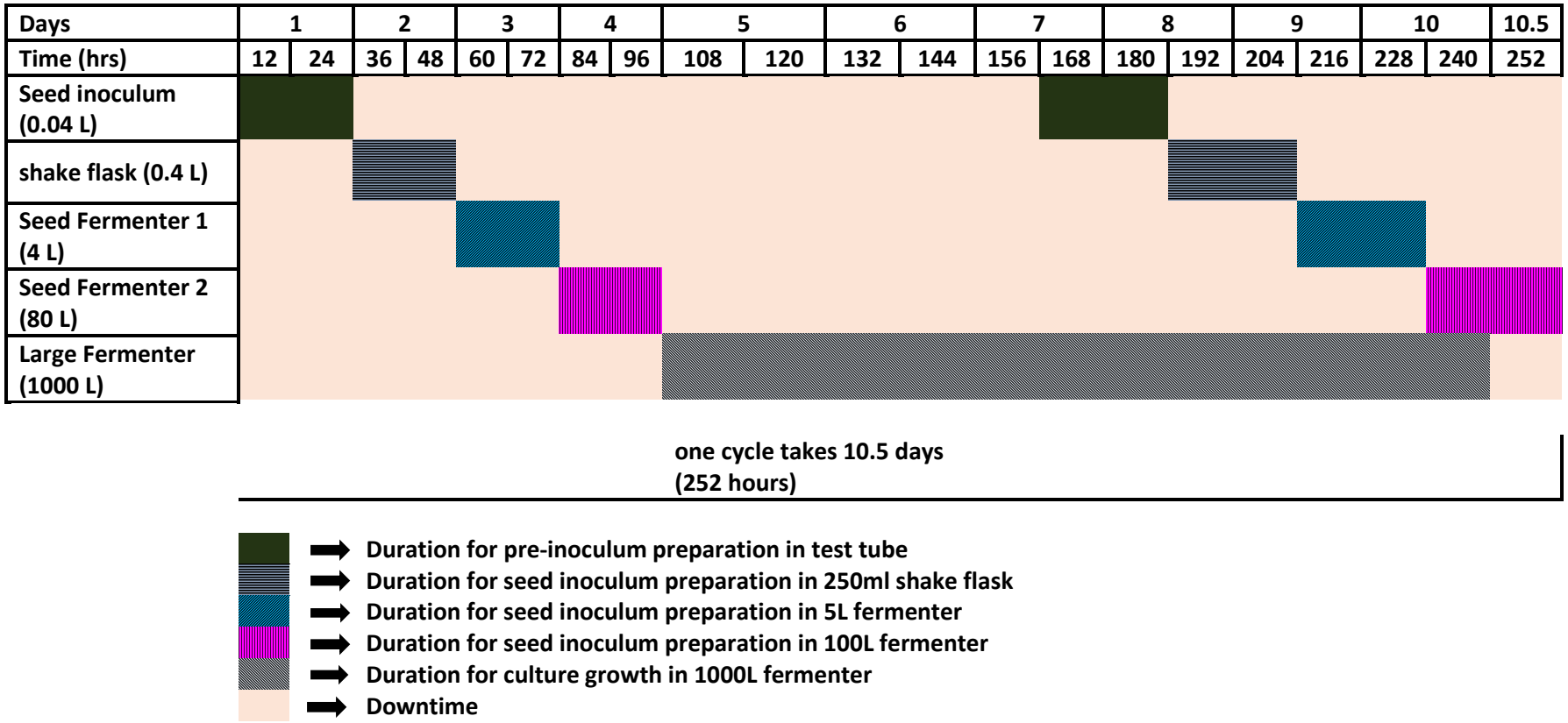

Figure S1: Production schedule for β-fructofuranosidase production in bioreactor

Appendix C: Supplementary information

Optimization of inulooligosaccharides production from inulin substrates extracted from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers

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Table S1: Experimental design for combination of independent variables at different levels using CCD and RSM

Run	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9 (C)**	**10 (C)**	**11 (C)**	**12 (C)**
Time, h	2.0	2.0	6.0	6.0	1.2	6.8	4.0	4.0	4.0	4.0	4.0	4.0
Enzyme dosage, U/g_{inulin}	5.0	25.0	5.0	25.0	15.0	15.0	0.8	28.7	15.0	15.0	15.0	15.0

Table S2: Carbohydrate composition of reaction products from enzymatic hydrolysis on JA powder

Time	**Percentage of sugars, % (w/w_{inulin})**												
	G	**F**	**GF**	**F2**	**GF2**	**F3**	**GF3**	**F4**	**GF4**	**F5**	**GF5**	**F6**	**GF6**	**IOS**
0	2.0	4.8	0.4	3.5	0.7	3.1	2.4	4.8	5.0	9.0	8.7	6.6	2.6	25.0
2	7.2	24.5	0.9	3.6	1.6	15.5	7.1	11.2	11.1	8.9	8.5	0.0	3.6	55.4
4	7.2	28.6	1.7	2.6	2.3	21.7	8.5	13.2	8.4	3.4	2.5	0.0	0.0	57.5
6	7.4	32.3	0.7	18.2	2.6	18.9	7.3	5.1	4.3	2.2	1.0	0.0	0.0	40.3
8	7.6	32.7	1.0	17.9	2.7	20.6	7.2	4.7	2.7	2.6	0.3	0.0	0.0	40.4
10	8.0	32.8	1.0	20.9	2.6	20.8	7.2	3.9	2.0	0.8	0.0	0.0	0.0	37.3
12	9.0	34.6	0.5	20.0	3.3	19.7	6.6	3.3	0.9	2.2	0.0	0.0	0.0	36.0

Table S3: Carbohydrate composition of reaction products from the various CCD runs on pure chicory inulin

Run	Percentage of sugars, % (w/w$_{inulin}$)													
	G	**F**	**GF**	**F2**	**GF2**	**F3**	**GF3**	**F4**	**GF4**	**F5**	**GF5**	**F6**	**GF6**	**IOS**
1	0.0	0.7	0.0	2.9	0.1	28.1	3.4	29.1	7.7	0.0	7.6	0.0	20.4	68.4
2	0.0	2.2	0.0	7.0	0.3	29.3	4.1	26.5	7.6	0.0	6.4	0.0	16.5	67.9
3	0.0	0.9	0.0	3.0	0.2	30.2	3.9	29.3	8.0	0.0	7.9	0.0	16.5	71.7
4	0.0	0.7	0.0	4.7	0.3	33.2	4.2	29.0	8.1	0.0	5.9	0.0	13.8	74.8
5	0.0	1.8	0.0	2.6	0.1	25.8	3.2	24.7	8.7	0.0	7.4	8.1	17.6	62.5
6	0.0	2.3	0.0	5.1	0.3	31.0	4.0	27.2	8.7	0.0	6.7	0.0	14.6	71.3
7	0.0	3.4	0.0	4.8	0.2	21.2	2.0	21.0	8.5	8.2	7.7	8.0	15.0	61.1
8	0.0	2.5	0.0	5.5	0.4	29.3	3.9	25.2	8.4	5.8	5.6	2.9	10.6	72.9
9 (C)	0.0	2.7	0.0	4.8	0.2	43.8	6.8	6.8	16.4	4.7	7.2	6.5	0.0	78.8
10 (C)	0.0	2.8	0.0	5.2	0.3	44.5	6.7	5.5	16.1	5.7	6.5	6.9	0.0	78.7
11 (C)	0.0	2.1	0.0	3.8	0.2	44.8	6.0	6.3	17.5	5.3	6.9	7.1	0.0	80.1
12 (C)	0.0	2.6	0.0	3.7	0.2	44.9	5.9	6.2	16.5	7.2	4.8	8.0	0.0	80.9

Table S4: Carbohydrate composition of reaction products from the various CCD runs on JA powder

Run	Percentage of sugars, % (w/w$_{inulin}$)													
	G	**F**	**GF**	**F2**	**GF2**	**F3**	**GF3**	**F4**	**GF4**	**F5**	**GF5**	**F6**	**GF6**	**IOS**
1	2.2	6.8	1.3	4.4	5.5	14.0	8.9	12.4	13.1	7.0	14.5	0.0	9.9	60.9
2	0.5	14.0	1.0	9.2	3.9	16.4	11.7	12.7	13.7	7.1	9.8	0.0	0.0	65.5
3	0.5	1.8	0.9	5.5	6.4	19.1	12.6	14.5	14.9	7.5	13.4	2.8	0.0	75.0
4	0.4	5.4	1.1	14.2	4.8	20.7	12.1	12.5	11.7	6.4	10.7	0.0	0.0	68.1
5	1.2	4.0	1.0	5.4	4.9	16.6	11.1	14.1	15.2	8.4	10.8	2.8	4.6	70.2
6	1.2	8.6	0.5	3.9	6.4	21.0	13.6	15.1	15.2	7.2	7.4	0.0	0.0	78.4
7	1.0	1.1	1.2	4.9	6.2	12.5	8.9	11.5	13.7	7.2	15.1	3.7	13.0	60.1
8	2.8	9.4	1.0	5.6	5.2	21.4	13.5	14.2	14.2	7.7	5.2	0.0	0.0	76.1
9 (C)	0.7	7.8	0.6	3.5	6.0	21.0	13.8	15.8	16.0	8.1	6.8	0.0	0.0	80.7
10 (C)	0.0	4.1	0.7	5.9	5.6	20.7	13.7	15.0	15.3	7.5	9.5	1.9	0.0	77.9
11 (C)	1.9	6.4	1.6	3.7	6.6	20.2	13.6	14.9	15.5	7.9	7.6	0.0	0.0	78.7
12 (C)	1.5	6.5	0.7	4.2	6.5	20.5	13.5	15.1	14.8	7.6	8.0	0.9	0.0	78.0

Table S5: Carbohydrate composition of reaction products from the various CCD runs on solid residue from JA

Run	Percentage of sugars, %(w/w$_{inulin}$)													
	G	**F**	**GF**	**F2**	**GF2**	**F3**	**GF3**	**F4**	**GF4**	**F5**	**GF5**	**F6**	**GF6**	**IOS**
1	5.1	16.0	1.0	6.2	2.3	19.6	7.4	17.7	11.2	6.8	6.7	0.0	0.0	65.0
2	5.9	17.3	1.1	8.1	1.8	20.9	7.5	17.3	9.5	4.8	6.0	0.0	0.0	61.7
3	4.4	12.8	0.9	6.8	2.7	24.9	10.1	18.9	11.3	4.4	3.2	0.0	0.0	72.1
4	6.9	21.0	1.7	12.8	2.3	23.8	7.8	14.0	7.3	0.0	2.4	0.0	0.0	55.2
5	5.4	16.1	1.0	4.7	2.0	19.9	6.8	18.6	10.6	6.9	8.0	0.0	0.0	64.8
6	4.8	23.1	1.6	10.5	2.9	19.9	8.7	15.4	9.9	0.0	3.2	0.0	0.0	56.9
7	6.2	32.3	2.7	15.1	3.2	9.9	4.5	8.2	7.4	4.1	6.5	0.0	0.0	37.2
8	4.5	30.8	3.0	15.9	6.1	12.5	6.1	7.8	12.2	0.0	1.3	0.0	0.0	44.6
9 (C)	4.5	14.2	0.9	6.8	2.3	25.2	9.2	20.1	10.6	2.8	3.5	0.0	0.0	70.1
10 (C)	3.9	12.4	0.9	6.7	2.8	25.4	10.9	20.9	9.4	3.4	3.4	0.0	0.0	72.8
11 (C)	3.6	13.3	0.7	5.9	1.8	26.7	9.3	22.8	8.2	4.4	3.3	0.0	0.0	73.2
12 (C)	1.9	12.9	0.8	9.3	1.6	25.6	10.5	21.0	8.6	4.9	2.8	0.0	0.0	72.3

Table S6: Carbohydrate composition of reaction products from the various CCD runs on inulin-rich extract from JA

Run	Percentage of sugars, %(w/w$_{inulin}$)													
	G	**F**	**GF**	**F2**	**GF2**	**F3**	**GF3**	**F4**	**GF4**	**F5**	**GF5**	**F6**	**GF6**	**IOS**
1	3.0	11.5	0.5	1.6	2.5	24.0	9.6	19.4	13.9	8.7	5.2	0.0	0.0	78.1
2	3.4	13.8	0.7	8.6	2.3	22.7	9.1	17.1	12.0	6.0	4.3	0.0	0.0	69.2
3	2.2	8.8	0.5	5.6	2.3	29.5	11.4	19.6	12.7	5.3	2.0	0.0	0.0	80.9
4	3.0	13.0	0.8	10.9	3.3	33.5	10.2	15.6	9.7	0.0	0.0	0.0	0.0	72.3
5	2.5	9.7	0.4	2.0	2.7	23.8	8.9	19.7	15.4	9.8	5.3	0.0	0.0	80.2
6	2.1	9.8	0.7	4.6	3.4	26.7	11.1	20.4	14.3	4.8	2.2	0.0	0.0	80.7
7	3.3	13.9	0.7	6.5	1.9	21.1	9.1	18.8	13.0	6.5	5.2	0.0	0.0	70.4
8	2.4	10.2	0.6	6.2	2.5	27.2	10.8	18.7	13.0	5.5	2.8	0.0	0.0	77.8
9 (C)	2.1	8.5	0.5	4.9	2.6	28.1	11.3	18.9	13.9	6.5	2.7	0.0	0.0	81.3
10 (C)	1.0	7.3	0.4	3.4	2.6	27.1	11.7	21.0	15.4	7.1	2.9	0.0	0.0	84.9
11 (C)	1.2	7.7	0.3	3.5	3.6	27.1	11.5	20.5	14.8	6.8	2.9	0.0	0.0	84.3
12 (C)	1.7	10.5	0.4	4.6	4.4	23.4	11.1	17.9	14.9	7.4	3.6	0.0	0.0	79.1

Appendix D: Supplementary information

Techno-economic analysis of inulooligosaccharides, protein and biofuel co-production from Jerusalem artichoke tubers in a biorefinery concept

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Table S1: Properties of the user-defined components used in the IOS production process

Component	Property	Quantity	Units	Comments
Protein	Formula	$CH_{1.57}O_{0.31}N_{0.29}S_{0.007}$		
	MW	22.8396	g/mol	
	DHSFRM	-17618	cal/mol	
Enzyme	Formula	$CH_{1.59}O_{0.42}N_{0.24}S0.01$		Properties were used to represent the β-fructofuranosidase enzyme.
	MW	24.0156	g/mol	
	DHSFRM	-17618	cal/mol	
GF2 and F3	Formula	$C_{18}H_{32}O_{16}$		The only available property data were the formula and MW. Their molecular structures were drawn in Aspen Plus which was used to estimate their thermodynamic properties.
	MW	504.438	g/mol	
GF3 and F4	Formula	$C_{24}H_{42}O_{21}$		
	MW	666.579	g/mol	
GF4 and F5	Formula	$C_{30}H_{52}O_{26}$		
	MW	828.727	g/mol	
Fibers	Used native Aspen component vanillin ($C_8H_8O_3$). The HHV of this compound (-23,906 BTU/kg) is very close to what we previously assumed for lignin as a custom component (-24,206)			
Inulin	Native Aspen Plus component cellulose was used to represent this component. DHSFRM = -233200.06 cal/mol			
Sources: Adopted from Humbird, D. et al., Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol, National Renewable Energy Laboratory (NREL), Golden, CO, 2011; http://mastersearch.chemexper.com				

Table S2: Stream table for scenario A

MATERIAL													
Name	**AIR**	**EXAIR**	**H2SO4**	**IOS**	**IOSOUT**	**JA**	**PROTEIN**	**RESIDUES**	**S12**	**S24**	**WATER**	**WATER2**	**WATER3**
Flow rate kg/hr	72729	76271	1	8	8	2285	78	263	260	4581	952	2039	2832
Temp ^{0}C	25	85	25	85	35	25	25	104	25	102	25	25	60
Pressure, bar	1	2	1	2	1	1	1	1	1	1	1	1	1
Mass fraction													
GLUCOSE	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000	0.000
WATER	0.015	0.061	0.000	0.030	0.030	0.800	0.479	0.346	0.900	0.999	1.000	1.000	1.000
CO2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ENZYME	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.068	0.100	0.000	0.000	0.000	0.000
ETHANOL	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SUCROSE	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.051	0.000	0.000	0.000	0.000	0.000
FRUCTOSE	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.126	0.000	0.000	0.000	0.000	0.000
NAOH	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H2SO4	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
CITRI-01	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
NA2HPO4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
O2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
AIR	0.985	0.939	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
N2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PROTEIN	0.000	0.000	0.000	0.000	0.000	0.030	0.486	0.018	0.000	0.000	0.000	0.000	0.000
FIBER	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.104	0.000	0.000	0.000	0.000	0.000
INULIN	0.000	0.000	0.000	0.001	0.001	0.130	0.034	0.028	0.000	0.000	0.000	0.000	0.000
CELLULOS	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.193	0.000	0.000	0.000	0.000	0.000

F2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.000	0.000	0.000	0.000	0.000
G	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	0.000	0.000	0.000	0.004	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF2(S)	0.000	0.000	0.000	0.071	0.071	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF3(S)	0.000	0.000	0.000	0.115	0.115	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F3(S)	0.000	0.000	0.000	0.105	0.105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F4(S)	0.000	0.000	0.000	0.219	0.219	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F5(S)	0.000	0.000	0.000	0.143	0.143	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F2(S)	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF4(S)	0.000	0.000	0.000	0.310	0.310	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Total	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table S3: Stream table for scenario B

MATERIAL											
Name	**AIR**	**EXAIR**	**H2OVAP**	**IOS**	**IOSOUT**	**JA**	**RESIDUES**	**S1**	**S12**	**WATER**	**WATER3**
Flowrate, kg/hr	68811	72835	5229	9	9	2165	251	5229	246	4325	2679
Temp, ^{0}C	25	64	104	64	35	25	104	102	25	25	60
Pressure, bar	1	2	1	2	1	1	1	1	1	1	1
Mass fraction											
GLUCOSE	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000
WATER	0.015	0.069	0.999	0.033	0.033	0.800	0.287	0.999	0.900	1.000	1.000
CO2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ENZYME	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.100	0.000	0.000
ETHANOL	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SUCROSE	0.000	0.000	0.000	0.000	0.000	0.000	0.043	0.000	0.000	0.000	0.000
FRUCTOSE	0.000	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.000	0.000
NAOH	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

H2SO4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
CITRI-01	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
NA2HPO4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
O2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
AIR	0.985	0.931	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
N2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PROTEIN	0.000	0.000	0.000	0.000	0.000	0.030	0.184	0.000	0.000	0.000	0.000
FIBER	0.000	0.000	0.000	0.000	0.000	0.014	0.086	0.000	0.000	0.000	0.000
INULIN	0.000	0.000	0.000	0.001	0.001	0.130	0.024	0.000	0.000	0.000	0.000
CELLULOS	0.000	0.000	0.000	0.000	0.000	0.026	0.160	0.000	0.000	0.000	0.000
F2	0.000	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000
G	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
GF	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
F	0.000	0.000	0.000	0.003	0.003	0.000	0.000	0.000	0.000	0.000	0.000
GF2(S)	0.000	0.000	0.000	0.072	0.072	0.000	0.000	0.000	0.000	0.000	0.000
GF3(S)	0.000	0.000	0.000	0.116	0.116	0.000	0.000	0.000	0.000	0.000	0.000
F3(S)	0.000	0.000	0.000	0.106	0.106	0.000	0.000	0.000	0.000	0.000	0.000
F4(S)	0.000	0.000	0.000	0.222	0.222	0.000	0.000	0.000	0.000	0.000	0.000
F5(S)	0.000	0.000	0.000	0.145	0.145	0.000	0.000	0.000	0.000	0.000	0.000
F2(S)	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000
GF4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GF4(S)	0.000	0.000	0.000	0.298	0.298	0.000	0.000	0.000	0.000	0.000	0.000
Total	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table S4: Stream table for scenario C

MATERIAL															
Name	**AIR**	**EXAIR**	**H2OVAP**	**H2SO4**	**IOS**	**IOSOUT**	**JA**	**PROTEIN**	**RESIDUES**	**S3**	**S12**	**S37**	**WATER**	**WATER2**	**WATER3**
Flowrate, kg/hr	75364	80114	6423	1	21	21	2368	81	258	6423	269	2821	987	2344	2640
Temp, ^{0}C	25	62	104	25	62	35	25	25	104	102	25	25	25	25	60
Pressure, bar	1	2	1	1	2	1	1	1	1	1	1	1	1	1	1
Mass fraction															
GLUCOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0295	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
WATER	0.0150	0.0734	0.9992	0.0000	0.0364	0.0364	0.8000	0.4786	0.3443	0.9992	0.9000	1.0000	1.0000	1.0000	1.0000
CO2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ENZYME	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0679	0.0000	0.1000	0.0000	0.0000	0.0000	0.0000
ETHANOL	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SUCROSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0496	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
FRUCTOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1218	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NAOH	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0013	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
H2SO4	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0013	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CITRI-01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NA2HPO4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF2	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000
GF3	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000
F3	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000
F4	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000
F5	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000
O2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AIR	0.9850	0.9265	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
N2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
PROTEIN	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0300	0.4864	0.0176	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
FIBER	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0140	0.0002	0.1044	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
INULIN	0.0000	0.0000	0.0000	0.0000	0.0391	0.0391	0.1300	0.0343	0.0345	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CELLULOS	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0260	0.0005	0.1939	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0336	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G	0.0000	0.0000	0.0000	0.0000	0.0008	0.0008	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF	0.0000	0.0000	0.0000	0.0000	0.0014	0.0014	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F	0.0000	0.0000	0.0000	0.0000	0.0034	0.0034	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF2(S)	0.0000	0.0000	0.0000	0.0000	0.0678	0.0678	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF3(S)	0.0000	0.0000	0.0000	0.0000	0.1095	0.1095	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F3(S)	0.0000	0.0000	0.0000	0.0000	0.1001	0.1001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

F4(S)	0.0000	0.0000	0.0000	0.0000	0.2090	0.2090	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F5(S)	0.0000	0.0000	0.0000	0.0000	0.1361	0.1361	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F2(S)	0.0000	0.0000	0.0000	0.0000	0.0009	0.0009	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF4	0.0000	0.0000	0.0003	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0003	0.0000	0.0000	0.0000	0.0000	0.0000
GF4(S)	0.0000	0.0000	0.0000	0.0000	0.2956	0.2956	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Total	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

Table S5: Summary of stream table for scenario D

MATERIAL													
Name	**AIR**	**BOIGAS**	**EXAIR**	**H2SO4**	**IOS**	**IOSOUT**	**JA**	**PROTEIN**	**S12**	**WATER**	**WATER2**	**WATER3**	**WWT**
Flowrate, kg/hr	72729	153	76271	1	8	8	2285	78	260	952	2039	2832	4808
Temp, ^{0}C	25	33	85	25	85	35	25	25	25	25	25	60	33
Pressure, bar	1	1	2	1	2	1	1	1	1	1	1	1	1
Mass fraction													
GLUCOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
WATER	0.0150	0.0377	0.0607	0.0000	0.0301	0.0301	0.8000	0.4786	0.9000	1.0000	1.0000	1.0000	0.9685
CO2	0.0000	0.4763	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0287
ENZYME	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1000	0.0000	0.0000	0.0000	0.0000
ETHANOL	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SUCROSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
FRUCTOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NAOH	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
H2SO4	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001
CITRI-01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NA2HPO4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

F5	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
O2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AIR	0.9850	0.0000	0.9392	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
N2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
PROTEIN	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0300	0.4864	0.0000	0.0000	0.0000	0.0000	0.0000
FIBER	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0140	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000
INULIN	0.0000	0.0000	0.0000	0.0000	0.0008	0.0008	0.1300	0.0343	0.0000	0.0000	0.0000	0.0000	0.0000
CELLULOS	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0260	0.0005	0.0000	0.0000	0.0000	0.0000	0.0000
F2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G	0.0000	0.0000	0.0000	0.0000	0.0009	0.0009	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF	0.0000	0.0000	0.0000	0.0000	0.0015	0.0015	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F	0.0000	0.0000	0.0000	0.0000	0.0036	0.0036	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF2(S)	0.0000	0.0000	0.0000	0.0000	0.0710	0.0710	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF3(S)	0.0000	0.0000	0.0000	0.0000	0.1147	0.1147	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F3(S)	0.0000	0.0000	0.0000	0.0000	0.1050	0.1050	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F4(S)	0.0000	0.0000	0.0000	0.0000	0.2190	0.2190	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F5(S)	0.0000	0.0000	0.0000	0.0000	0.1426	0.1426	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F2(S)	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF4(S)	0.0000	0.0000	0.0000	0.0000	0.3098	0.3098	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
METHA-01	0.0000	0.4854	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0015
ACETI-01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HYDRO-01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AMMON-01	0.0000	0.0006	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0006
SLUDGE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0006
GLYCI-01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GLYCI-02	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Total	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

Table S6: Stream table for scenario E

MATERIAL													
Name	**AIR**	**CO2**	**ENZYME**	**ETOH**	**EXAIR**	**H2OVAP**	**H2SO4**	**IOS**	**IOSOUT**	**JA**	**L409**	**L411**	**NS6**
Flowrate, kg/hr	72729	83	14	85	76257	4551	1	262	262	2857	416	207	83
Temp, ^{0}C	25	30		30	86	104	25	86	35	25	105	129	112
Pressure, bar	1	1	1	1	2	1	1	2	1	1	3	3	2
Mass fraction													
GLUCOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0247	0.0000	0.0000
WATER	0.0150	0.0157	0.0000	0.0035	0.0605	0.9988	0.0000	0.0299	0.0299	0.8000	0.3028	0.5890	0.9991
CO2	0.0000	0.9836	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0688	0.0000	0.0000
ENZYME	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2564	0.0000	0.0000
ETHANOL	0.0000	0.0007	0.0000	0.9965	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2053	0.4110	0.0009
SUCROSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
FRUCTOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0198	0.0000	0.0000
NAOH	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
H2SO4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000	0.0012	0.0000	0.0000
CITRI-01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NA2HPO4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F5	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
O2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AIR	0.9850	0.0000	0.0000	0.0000	0.9394	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
N2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
PROTEIN	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0300	0.0162	0.0000	0.0000
FIBER	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0140	0.0960	0.0000	0.0000
INULIN	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0008	0.0008	0.1300	0.0000	0.0000	0.0000
CELLULOS	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0260	0.0089	0.0000	0.0000

F2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0009	0.0009	0.0000	0.0000	0.0000	0.0000
GF	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0015	0.0015	0.0000	0.0000	0.0000	0.0000
F	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0036	0.0036	0.0000	0.0000	0.0000	0.0000
GF2(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0710	0.0710	0.0000	0.0000	0.0000	0.0000
GF3(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1147	0.1147	0.0000	0.0000	0.0000	0.0000
F3(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1050	0.1050	0.0000	0.0000	0.0000	0.0000
F4(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2191	0.2191	0.0000	0.0000	0.0000	0.0000
F5(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1427	0.1427	0.0000	0.0000	0.0000	0.0000
F2(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0000	0.0000	0.0000
GF4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF4(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.3099	0.3099	0.0000	0.0000	0.0000	0.0000
Total	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

Table S7: Stream table for scenario E (continued)

MATERIAL														
Name	**NS7**	**NS12**	**NS15**	**PROTEIN**	**RESIDUES**	**S12**	**S18**	**S30**	**SLDWASTE**	**WATER**	**WATER2**	**WATER3**	**WWT**	**YEAST**
Flow rate, kg/hr	85	124	20	162	59	260	5	4551	180	952	2039	2832	83	14
Pressure, ^{0}C	93	103	25	25	93	25	25	102	121	25	25	60	112	
Pressure, bar	1	2	1	1	1	1	2	1	6	1	1	1	2	1
Mass fraction														
GLUCOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0570	0.0000	0.0000	0.0000	0.0000	0.0000
WATER	0.0035	0.3156	1.0000	0.4786	0.9985	0.7000	1.0000	0.9988	0.0224	1.0000	1.0000	1.0000	0.9991	0.0000
CO2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ENZYME	0.0000	0.0000	0.0000	0.0000	0.0000	0.3000	0.0000	0.0000	0.5924	0.0000	0.0000	0.0000	0.0000	1.0000
ETHANOL	0.9965	0.6844	0.0000	0.0000	0.0014	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0009	0.0000
SUCROSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
FRUCTOSE	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0457	0.0000	0.0000	0.0000	0.0000	0.0000
NAOH	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

H2SO4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0028	0.0000	0.0000	0.0000	0.0000	0.0000
CITRI-01	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NA2HPO4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F5	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
O2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AIR	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
N2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
PROTEIN	0.0000	0.0000	0.0000	0.4864	0.0000	0.0000	0.0000	0.0000	0.0374	0.0000	0.0000	0.0000	0.0000	0.0000
FIBER	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.2218	0.0000	0.0000	0.0000	0.0000	0.0000
INULIN	0.0000	0.0000	0.0000	0.0343	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CELLULOS	0.0000	0.0000	0.0000	0.0005	0.0000	0.0000	0.0000	0.0000	0.0206	0.0000	0.0000	0.0000	0.0000	0.0000
F2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF2(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF3(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F3(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F4(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F5(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F2(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GF4(S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Total	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

Table S8: Estimations of FCI, TOC and MSP for the various biorefinery scenarios at production scales of 2000, 5000 and 10000 tpa IOS

	Scenario A			Scenario B			Scenario C			Scenario D			Scenario E		
Capacity, tpa	FCI, M$	TOC, M$	MSP, $/kg	FCI, M$	TOC, M$	MSP, $/kg	FCI, M$	TOC, M$	MSP, $/kg	FCI, M$	TOC, M$	MSP, $/kg	FCI, M$	TOC, M$	MSP, $/kg
2 000	48.7	5.5	4.6	36.0	5.2	3.9	52.8	5.7	5.2	61.9	6.1	6.5	68.4	6.9	7.4
5 000	84.5	13.7	3.6	62.3	12.9	3.2	91.5	14.4	4.1	107.3	15.3	5.2	118.5	17.2	6.0
10 000	128.0	27.5	3.0	94.5	25.9	2.7	138.6	28.7	3.5	162.6	30.6	4.5	179.7	34.5	5.2